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13. ABSTRACT (Maximum 200 Words)

The purpose of this research project is to investigate the interaction between heat shock protein 27 (hsp27) and cytochrome c in the inhibition of apoptosis. The scope of the study was to include: measuring the binding of hsp27 to cytochrome c in vivo, determining why hsp27 binds to cytochrome c and determining the fate of the hsp27:cytochrome c complex. Major findings include cellular survival curves to cytochrome c, ATP, FAD and NAD. All electron carriers were cytotoxic to human breast cancer cell lines, with FAD being the most cytotoxic. This finding could have applications in the treatment of breast cancer. Molecular chaperone assays with hsp27 and alphaglucosidase and citrate synthase indicate that hsp27 binds to cytochrome c in a non-specific manner.

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Introduction

This is the final report for the concept award DAMD17-01-1-0571 that covers the period from June 2001 through May 2003. The concept award is typically for a one-year period, but a one-year time extension was granted for this project. The objectives of the proposed study were to (1) measure the binding of hsp27 to cytochrome c in vivo, (2) determine why hsp27 binds to cytochrome c and (3) determine the fate of the hsp27:cytochrome c complex.

Body

This report will refer to material found in the Appendices. Appendix A contains a manuscript entitled "Hsp27 Inhibits Apoptosis by Binding to Cytochrome c in Human Breast Cancer Cells". Appendix B contains a figure demonstrating that caspase 9 activation is required for caspase 3 activation in cell free extracts. Appendix C contains a rough draft of a thesis entitled "Cytochrome c can overcome Hsp27 Inhibition of Apoptosis in Human Breast Cancer cells". Appendix D contains the material and methods and results sections from a manuscript that is in preparation. Appendix E contains a honors thesis entitled "Inhibition of Apoptosis by elevated levels of Heat Shock Protein 27 in Human Breast Cancer Cells".

To begin the first objective, we started with cell free extracts and demonstrated that the addition of cytochrome c was sufficient to activate caspase 3 activity (figure 5, Manuscript, Appendix A). We next wanted to demonstrate that hsp27 could bind to cytochrome c and inhibit the activation of caspase 3. Figure 6 (Manuscript, Appendix A) clearly shows hsp27 can indeed inhibit the activation of caspase 3 in vitro. To help confirm that hsp27 is not directly inhibiting caspase 3, we investigated the ability of hsp27 to inhibit recombinant caspases. Table 1 (Manuscript, page 23, Appendix A) demonstrates that hsp27 is not able to inhibit caspase 3, 8 or 9. To further confirm cytochrome c is involved in apoptosome formation, which leads to caspase 9 activation, and hence caspase 3 activation, we added cytochrome c to a cell free extract in the presence or absence of a caspases 9 inhibitor. The data shown in figure 10 (Appendix B) demonstrates that as expected caspase 9 plays a major role in the activation of caspase 3.

To directly demonstrate that hsp27 and cytochrome c forms a complex, a series of immunoprecipitation experiments were performed. Figure 4 (Manuscript, Appendix A) demonstrates that when cell extracts are immunoprecipitated with an anticytochrome c antibody, the precipitate contains hsp27 even though there is no cross reactivity between the antibody and hsp27. Figure 4 also demonstrates that when cells extracts are immunoprecipitated with an antihsp27 antibody, the precipitate contains cytochrome c, even though there is no cross reactivity between the antibody and cytochrome c.

To continue our studies on the interactions between hsp27 and cytochrome c, we added cytochrome c to our engineered breast cancer cell lines. Figure 8(Manuscript, Appendix A) shows that cytochrome c is toxic and that cells that express elevated levels of hsp27 are better able to survive the cytotoxic effects of cytochrome c. We also demonstrated that cytochrome c was toxic to other breast cancer cell lines including MCF-7 and MDA-MB-435, Figure 5 (Thesis, page 30, Appendix C).

We interpreted this result to indicate that cytochrome c was taken up by the cell and thus triggered apoptosis. As a control to help confirm this interpretation, we added another electron carrier, FAD. Figure 7 (Thesis, page 32, Appendix C) shows that FAD is cytotoxic at low concentrations. Figure 8 (Thesis, page 33, Appendix C) shows that both MCF-7 and MDA-MB-435 cell lines are also sensitive to FAD. When FAD was added at the same concentrations as cytochrome c, even greater cell killing was observed, Figure 15 (Thesis, page 41, Appendix C).

NAD was also efficient at killing breast cancer cells, Figure 11 (Thesis, page 36, Appendix C). Therefore, it appears that most electron carriers when added exogenously to human breast cancer cells are able to induce cell death. As far as we can determine, this is the first report of electron carriers (other than cytochrome c) being cytotoxic to breast cancer cells.

While we have not been able to directly demonstrate that cytochrome c enters the cell, we have shown that other electron

carriers are capable of inducing apoptosis in a variety of human breast cancer cell lines. Therefore, the cytochrome c effects may be due to its ability to transfer electrons as opposed to its ability to form an apoptosome. We plan on continuing this line of investigation to determine the mechanism that these electron carriers are using to induce apoptosis, since this observation has clinical applications in the treatment of breast cancer.

To begin the second objective, we investigated the molecular chaperone ability of hsp27. Since cytochrome c does not have an enzymatic activity we decided to focus initially on well-characterized enzymes, which have been used in other molecular chaperone studies. The first enzyme we investigated was alphaglucosidase. Figure 1 (Appendix D) demonstrates that the activity prior to heating was 100% and that following heating it fell to 32%. If heating (45°C, three minutes) was conducted in the presence of hsp27 more activity was preserved. If the experiment was conducted with aldolase in place of hsp27, no protection was observed. Hsp27 provided this protection in the absence of ATP. Other molecular chaperones such as Hsp70 and GroEL/GroES will provide protection but require ATP to do so (data not shown).

The next enzyme we investigated was citrate synthase. Figure 5 (Appendix D) shows that following heating citrate synthase activity fell from 100% to 40%. If hsp27 was added prior to heating it could protect some of the enzymatic activity (about 30%) but a control protein, IgG, could not. Figure 5 also shows that if hsp27 is added following the heating, it can not help citrate synthase recover any enzymatic activity. We interpret these results to indicate that as a protein denatures, it can bind to hsp27 (most likely through newly exposed hydrophobic sites), which then prevents the protein from aggregating and thus loosing function. We predict that as cytochrome c exits the mitochondria it is partially denatured and thus able to bind to hsp27. Once bound, the cytochrome c in unavailable to bind to the other components of the apoptosome and thus activate the caspase cascade of apoptosis.

Since both alphaglucosidase and citrate synthase gave similar results it was decided not to extent these studies to a third enzyme DHFR.

The third specific objective, determine the fate of the hsp27:cytochrome c complex, has been slow due to technical problems of detecting the complex. We initially investigated a SuperRose Grade 6 column due to its ability to resolve large protein complexes. However, as can be seen in figure 7 (Appendix E, page 10) the resolving ability of this column in our hands was not suitable. We next investigated two SuperDex 2000 columns, which should result in placing the apoptosome in the void volume and the component proteins (i.e. cytochrome c, Apaf-1 and procaspase 9) in various column fractions. Figure 11 and 12 (Appendix E, pages 11 & 12) shows that the SuperDex columns were able to resolve our protein standards: thyroglobulin (669 kDa), apoferritin (443 kDa), bovine serum albumin (67 kDa) and lysozyme (27 kDa). Both SuperDex columns (labeled B & C) had identical elution patterns of the protein standards, figure 13 (Appendix E, page 12) and showed a linear relation between the log of the molecular weight vs. fraction number, figures 14 & 15 (Appendix E, page 12). When a cell free extract from our control transfected cell line (DC4) was applied to the column and the fractions assayed for caspase 3 activity, no activity was detected, figure 18 (Appendix E, page 14). However, if this cell line was exposed to 10 mM sodium butyrate for 15 minutes prior to harvesting, the extract had a peak of caspase 3 activity in fraction #32 which corresponds to a molecular weight of approximately 120 kDa (figure 19, Appendix E, page 15). This size is consistent with a dimmer of caspase 3. When a cell free extract from the cell line DB46 (which constitutively expresses hsp27) was applied to the SuperDex column, a slight peak of activity was observed in fraction #24, figure 22, (Appendix E, page 16). The peak at fraction #24 corresponds to a molecular weight of 190kDa, the size of a caspase 9 dimer. Following incubation with 10 mM Sodium butyrate, the cell free extract also had a small peak of caspase 3 activity in fraction #34, figure 23 (Appendix e, page 16). Since the caspase 3 peak acitivity in DB46 cells is smaller then the caspase 3 peak activity in DC4 cells, we interpret this observation to indicate that the DC4 cells had more cytochrome c available to form apoptosomes which activated more caspase 3 molecules.

We did not further characterize (i.e. Western Blots, caspase 9 activity assays) the fractions from the SuperDex columns. Instead

we purchased an AKTA FPLC system from Amersham about six months ago. This system can now give us very reproducible results. We believe we have identified the conditions necessary for the separation of the apoptosome in a cell free extract. Our current conditions are a SuperRose 6 column, with a 0.2 ml/min flow rate of a buffer containing 40 mM Hepes (pH 7.5, KOH), 10 mM KCl and 1 mM Dithiothreitol. We were unable to complete this objective in the time allowed but we plan on continuing this project until the objectives are achieved as we now have the necessary technology in place.

Key Research Accomplishments:

Cellular Survival Curves to:
- Cytochrome c
- FAD
- NAD
- ATP

Hsp27 Molecular Chaperone Assays with:
- Citrate Synthase
- Alpha-Glucosidase

Reportable Outcomes:

Invited Presentation. S.W. Carper. "The role of heat shock proteins in the inhibition of apoptosis". Radiation Research Society, 2002, Reno, NV.

Poster Session. S.W. Carper. "Heat Shock Protein 27 Inhibits Apoptosis in Human Breast Cancer cells by Binding to Cytochrome c". Era of Hope U.S. Army Breast Cancer Research Conference, 2002, Orlando, Fl.

Poster session "Heat Shock Protein 27 Can Protect against Apoptosis Induced by Electron Carriers in Human Breast Cancer Cells" accepted to the XIX International Congress of Biochemistry and Molecular Biology during July 2003 in Toronto Canada. Unfortunately, this meeting was canceled due to the SARS outbreak.

Thesis "Cytochrome c can overcome Hsp27 inhibition of Apoptosis in Human Breast Cancer cells", Yvonne Giraud, UNLV 2003.

Manuscript "Hsp27 Inhibits Apoptosis by Binding to Cytochrome c in Human Breast Cancer Cells, Lewis Joe Stafford, Yvonne Giraud, and Stephen W. Carper, to be submitted to Cancer Research 7/03.

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Hokuna Keehu
Yvonne Giraud
John Stiever

Conclusions:

In summary, we have been able to show that hsp27 forms a complex with cytochrome c which inhibits apoptosome activity from activating caspase 3. The interaction between hsp27 and cytochrome c is apperntly not specific, since hsp27 can bind to and protect the enzymatic function of both alphaglucosidase and citrate

synthase. The most interesting finding has been that electron carriers FAD and NAD are both cytotoxic to human breast cancer cells. This observation could have important clinical applications in the treatment of breast cancer. This compounds are inexpensive and cytotoxic at very low doses to tissue culture cells. If these observations are confirmed in future animal models, then human testing would be justified.

References: Included in the Appendices

Appendix A

HSP27 Inhibits Apoptosis by Binding to Cytochrome c in Human Breast Cancer Cells

Hsp27 Inhibits Apoptosis by Binding to Cytochrome c in Human Breast Cancer Cells

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Abstract

Hsp27 is a molecular chaperone that can protect cancer cells from a variety of stresses that are known to induce apoptosis. To better define the mechanism of action of hsp27 in the inhibition of apoptosis, two genetically engineered human breast cancer cell lines were used that differ only in the constitutive level of hsp27 expression. Constitutive expression of hsp27 indirectly inhibited activation of caspase 9 and caspase 3, but hsp27 did not directly inhibit these enzymes once they were activated. The inhibition of activation was a direct result of hsp27 binding to cytochrome c which formed a stable complex that was immunoprecipitated by using either an anti-hsp27 antibody or an anti-cytochrome c antibody. Cytochrome c was able to activate caspase 3 in a cell free extract but incubation of hsp27 with cytochrome c inhibited caspase 3 activation. Granzyme b was able to activate caspase 3 in the cell free extract in the presence or absence of hsp27. Constitutive expression of hsp27 was able to protect cell from the cytotoxic effects of cytochrome c added exogenously. These results clearly indicate that hsp27 inhibits apoptosis by binding to cytochrome c.

Introduction

Apoptosis (programmed cell death) is a process whereby specific proteins become activated leading to cell death characterized by distinctive events including nucleosome degradation and packaging, formation of apoptotic vesicles and appearance of phosphatidylserine in the outer envelope of the lipid bilayer (reviewed in Wolf and Green 1999, Kromer and Reed 2000, Zimmermann, et al., 2001). Apoptosis can be induced through receptor mediated events (i.e. Fas, TNF) or through an internal signaling process involving the release of cytochrome c from mitochondria. Receptor mediated apoptosis involves a ligand which causes the assembly of its homotrimeric receptor. Adaptor molecules such as FADD or DAXX then bind the receptor through their death domains (Chinnaiyan et al., 1995). The adapter molecules then activate procaspase 8 to caspase 8 (FADD) or activate kinases (DAXX). Once activated, caspase 8 can proceed to activate downstream caspases 7 & 3 which ultimately cleave targets resulting in the classical hallmarks of apoptosis. Mitochondria mediated apoptosis (internal signaling) involves a stress to the cell that signals to the mitochondria to release cytochrome c through the permeability transition pore. Once released, cytochrome c recruits Apaf-1, procaspase 9 and dATP to form a large (approximately 700 kd) structure known as the apoptosome (Cain et al., 1999). The apoptosome then activates procaspase 9 to caspase9 activating downstream caspase 3.

Typically, cells can be induced to undergo apoptosis and normally do so as a mechanism to remove damaged or infected cells in a population. However, most cancer cells do not undergo apoptosis. This inhibition of apoptosis can be the result of an increased expression of a number of proteins known to inhibit apoptosis. One group of proteins that have recently been reported to inhibit apoptosis is the heat shock proteins. These proteins demonstrate an increase in expression following a cellular stress (including heat shock) and are thought to be able to protect the cell from subsequent stresses. Most heat shock proteins are also recognized as molecular chaperones (reviewed in Nollent & Morimoto 2002 and Haslbeck & Buchner 2002). Most human cells express heat shock proteins (hsps) with molecular weights of 110, 90, 70 and 27 kilodaltons. Most of these hsps are members of multigene families, some of which are expressed constitutively while others are induced by stress.

Heat shock protein 27 (hsp27) is inducible by a variety of stresses as well as by estrogen. This protein can help prevent other proteins from denaturing or keep them in a refoldable state (Jackob et al., 1993) In the cytoplasm hsp27 forms a large aggregate (usually a 16-mer) which can increase in size following a heat shock. Constitutive expression of hsp27 has been reported to provide protection against a variety of stressors that induce apoptosis (reviewed in Concannon et al., 2003). The exact mechanism whereby hsp27 inhibits apoptosis remains unclear. Several proteins have been shown to inhibit apoptosis. The receptor mediated apoptosis induced by Fas or TNF, can be inhibited by XIAP, Crm A, or FLIP which inhibit the formation or activation of

receptor complexes. Mitochondrial mediated apoptosis can be inhibited by high levels of Bcl-2, which prevents the release of cytochrome c (Adams and Cory 1998). Recently hsp70 and hsp90 have been reported to inhibit the apoptosome formation by binding to Apaf-1 or procaspase 9, respectively (Beere et al., 2000, Pandey et al., 2000). Other proteins (CrmA, IAPs, Serpins, etc.) inhibit apoptosis induced by either receptor mediated or mitochondrial mediated processes by directly inhibiting activated caspases (reviewed in Deveraux et al., 1999 and Ye and Goldsmith, 2001). Recently, hsp27 has been reported to inhibit apoptosis by a receptor mediated pathway (binding to DAXX) and by a mitochondrial mediated pathway (binding to cytochrome c) (Bruey et al., 2000).

In this study hsp27 inhibited apoptosis in human breast cancer cells from a variety of stressors. The inhibition prevents the activation but not the activity of caspase 9 and caspase 3. Hsp27 binds to cytochrome c *in vitro* preventing the activation of caspase 3. This inhibition can be overcome by Granzyme b. Finally, this study demonstrates that cells constitutively expressing hsp27 are better able to survive apoptosis induced by the addition of cytochrome c.

Results

The ability of hsp27 to protect human breast cancer cells against agents known to induce apoptosis is shown in Figure 1. At the indicated concentration there was a statistically

significant protection in DB46 cells against apoptosis inducing agents. To better define the molecular step at which hsp27 was inhibiting apoptosis, the activation of specific caspases was evaluated.

Figure 2 indicates that caspase 3 is not activated in DB 46 cells following exposure to sodium butyrate, however, there is a distinct activation of caspase 3 in the DC 4 cell line. The only difference between these two cell lines is the constitutive expression of hsp27 in the DB 46 cell line (Rust et al., 1999). Similar results for caspase 9 are seen in Figure 3. The DB 46 cell line fails to show any activation of caspase 9 while the DC 4 cell line shows clear activation. These results suggest that hsp27 might inhibit activated caspases. To evaluate this mechanism the activity of recombinant caspase 3, 8 and 9 was measured in the presence and absence of hsp27 (Table 1). When hsp27 was added in equimolar amounts to the caspases no inhibition of activated caspases occurred. Therefore, the lack of caspase 3 and caspase 9 activity seen in DB46 cells (Figures 2 & 3) is due to an inability of the caspases to be activate but not a direct inhibition of caspase activity.

To further investigate the site of hsp27 inhibition immunoprecipitation experiments were conducted to determine if hsp27 was able to bind to cytochrome c. DC4 cell extracts were prepared as outlined in the materials and methods section. To these extracts were added either cytochrome c, hsp27 or both proteins. The extracts were either immunoprecipitated with an

anitcytochrome c antibody, proteins separated by SDSPAGE and analyzed by Western analysis using an antihsp27 antibody (Figure 4A) or immunoprecipitated with an antihsp27 antibody, proteins separated by SDSPAGE and analyzed by Western analysis using an anticytochrome c antibody (Figure 4B). In Figure 4A, lane 1 is an authentic sample of hsp27 loaded directly on the gel and then probed with the antihsp27 antibody. Lane 2 demonstrates that the antihsp27 antibody can immunoprecipitate hsp27. Lane 3 shows that the anticytochrome c antibody does not immunoprecipitate hsp27. Lane 4 shows that the DC4 cell extract contains an endogenous hsp27:cytochrome c complex. Lane 5 demonstrates the presence of endogenous hsp27 in the DC4 cell extracts. Lanes 6 & 7 demonstrates that the addition of cytochrome c to hsp27 results in an increase in the hsp27:cytochrome c complex (i.e. lane 6 shows a larger band than lane 7). In Figure 4B, Lane 1 is an authentic sample of cytochrome c loaded directly on the gel and then probed with the anticytochrome c antibody. Lane 2 demonstrates that the anticytochrome c antibody can immunoprecipitate cytochrome c. Lane 3 shows that the antihsp27 antibody does not immunoprecipitate cytochrome c. Lane 4 demonstrates that the DC4 cell extract contains an endogenous hsp27:cytochrome c complex. Lane 5 demonstrates the presence of endogenous cytochrome c in the DC4 cell extract. Lanes 6 &7 demonstrate that the addition of hsp27 to cytochrome c results in an increase in the hsp27:cytochrome c complex (i.e. lane 6 shows a larger band than lane 7). Figures 4A and 4B clearly show that a hsp27:cytochrome c complex is formed.

To demonstrate that the hsp27:cytochrome c complex has a biological function, the cell extracts from DC 4 cells were used again. The ability of exogenously added cytochrome c to stimulate caspase 3 (DVEDase) is shown in figure 5. There is an increase in caspase 3 activity following the addition of cytochrome c in a dose dependent manner. This is due to cytochrome c causing the formation of the apoptosome which activates caspase 9 which then results in the activation of caspase 3. To confirm this pathway, an inhibitor of caspase 9 was able to significantly inhibit the activation of caspase 3 (data not shown). In these extracts, five μg of cytochrome c consistently resulted in the greatest caspase 3 activity, while 10 μg of cytochrome c resulted in less activity. If cytochrome c is incubated with hsp27 prior to addition to the cell extract, there is a significant reduction in the ability of cytochrome c to simulate caspase 3 activation (figure 6).

If hsp27 inhibits apoptosis by binding to cytochrome c, then it should still be possible to activate caspase 3 in the presence of hsp27 if there is a direct activator. Granzyme b is a serine protease that is known to directly activate all caspases (Kam et al., 2000). Figure 7 shows that hsp27 is able to inhibit cytochrome c activation of caspase 3 but is unable to inhibit granzyme b action of caspase 3.

While our results demonstrate that hsp27 can interact with cytochrome c, these experiments have been conducted *in vitro* at concentrations that may exceed the normal cellular levels for cytochrome c and hsp27. To begin to demonstrate that hsp27 and cytochrome c interact *in vivo*,

we added cytochrome c to our breast cancer cell lines. Figure 8 shows that the exogenous addition of as little as 5.0×10^{-7}M cytochrome c to cells can induce cell death. However, at this concentration, no death is observed in the cell line that constitutively expresses hsp27. At higher concentrations of cytochrome c, both cell lines demonstrate cell death, however, constitutive expression of hsp27 always protected the DB46 cells resulting in a greater survival. This result suggests that hsp27 can protect human breast cancer cells from cytochrome c initiated apoptosis.

Discussion

Our results demonstrate that constitutive expression of hsp27 can protect human breast cancer cells from a wide variety of stressors known to induce apoptosis. This is in agreement with most published accounts of hsp27 function (Huot et al., 1991, Mehlen et al., 1996, Wagstaff et al., 1999, Bruey et al., 2000). We also demonstrated that hsp27 inhibits the activation of caspases 3 & 9 but does not directly inhibit caspases. This observation is in agreement with (Bruey et al., 2000) but is not consistent with (Voorter et al., 1994) who reported that the mouse small heat shock protein hsp25 and alpha-crystallin (which is strucullay related to the small heat shock proteins[Merck et al., 1993]) can inhibit elastase, a serine proteases. In our laboratory, hsp27 is not a good inhibitor for cystein type protesases. Figure 4 clearly shows that the endogenous

levels of hsp27 and cytochrome c in the control transfected cell line are sufficient to form a hsp27:cytochrome c complex that can be immunoprecipitated by either an antihsp27 antibody or an anticytochrome c antibody. The ability of hsp27 to form a complex with cytochrome c has been previously reported (Bruey et al., 2000) and is in agreement with our observations. The ability of hsp27 to inhibit cytochrome c induced caspases 3 (DEVDase) *in vitro* activity is in good agreement with Samali (Samali et al., 2001). A previous report of the ability of granzyme b to overcome this inhibition is in agreement with our observations (Bruey et al., 2000). The inhibition of DEVDase activation by hsp27 *in vitro* while interesting may be due to the ability of hsp27 at act as a molecular chaperone. It is well known hsp27 can bind to a variety of denatured/misfolded proteins (Jakob et al., 1993, Ehrnsperger et al., 1997). Perhaps in the *in vitro* assay hsp27 binds to cytochrome c because it is denatured or misfolded while under physiological conditions it would not do so. To begin to address the physiological relevance of hsp27/cytochrome c binding, we added cytochrome c to cell cultures to induce apoptosis. Figure 8 clearly shows that hsp27 is capable of protecting human breast cancer cells from the cytotoxic effects of exogenously added cytochrome c. This is the first report of hsp27 mediating protection of cells from cytochrome c *in vivo*.

A recently published report indicates that hsp27 can inhibit apoptosis by binding to the receptor adapter protein DAXX (Charette et al., 2000). DAXX is thought to function by activating the Ask1 kinase which can ultimately lead to apoptosis. The authors also reported that hsp27

expression had no effect on caspase-dependent apoptosis. This observation is not consistent with the results we report nor with earlier reports (Bruey et al., 2000). These differences could be due to the cell lines involved (human embronic kidney cells vs. human breast cancer cells) and/or the agents used to induce apoptosis.

In the past several years, reports have appeared that indicate hsp90, hsp70 and hsp27 can each inhibit apoptosis by binding to components of the apoptosome. The apoptosome forms when cytochrome c, Apaf-1, Procaspase 9 and dATP self assemble. Following formation, the apoptosome facilitates the activation of procaspase 9 to caspase 9. Hsp90 and hsp70 can bind to Apaf-1, a large cytosolic protein essential for apoptosome function, and inhibit cytochrome c mediated apoptosis (Pande et al., 2000, Beere et al., 2000). Hsp27 can bind to cytochrome c, which inhibits the formation of the apoptosome (Bruey at al. 2000 and this paper). It would appear that there exits multiple ways in which to inhibit the apoptotic pathway to allow cells to recover from "sub-lethal" stresses. The overlapping role of hsps in inhibiting apoptosis would explain many years of observations that hsps, either in groups (i.e. expressed in thermotolerant cells) or individually (i.e. genetically engineered cell lines) can provide protection against a wide variety of cellular stressors. This observation also suggests that hsps may serve as therapeutic targets in the treatment of cancer cells. Recently, in phase I clinical trails using geldanamycin, which is an inhibitor of the ATPase activity of hsp90, promising results have been seen (Neckers 2002). Hsp90 is know to interact with several oncogene products including v-Src, Bcr-Abl, Raf-

1, ErbB2 and some growth factor receptors and steroid receptors (Blagosklonny 2002). It is interesting to speculate that with decreased activity of this hsp the cancer cells may be more susceptible to undergoing apoptosis (vanden Berghe et al., 2003 and Fujita et al., 2002). Agents designed to decrease the levels of hsp70 and hsp27 may also prove effective in increasing a cell cells ability to undergo apoptosis.

Materials & Methods

Cell Lines. Stable transfected cell lines (DC4 & DB46, derived from an estrogen receptor-negative human breast cancer cell line MBA-MD-231) were isolated and grown in Minimal Essential Media (MEM) (Gibco BRL), supplemented with 10% fetal bovine serum, 25mM HEPES buffer, 100 IU penicillin/ml, 100 µg/ml streptomycin, 2 mM L-glutamine (Biowhittaker Inc.) and 600 µg/ml G418 sulphate (Gibco BRL). The constitutive expression vector pB27 was used in constructing DB 46 cells whereas the control vector pHB-Apr-1-neo was used in constructing DC 4 cells (Mahvi et al.,, 1996). All cultures were maintained at 37°C in humidified incubators with 5% CO_2:95% air. Twenty-four hours before an experiment, the cells were placed in MEM lacking G418.

Drugs and Proteins. Stock solutions of Vitamin E Succinate (VES), cyclohexamide (CHX) sodium butyrate (NaB) and cytochrome c (all from Sigma Chemical Co.) were prepared in

deionized water and filter sterilized (0.2 filter). HSP 27 was purchased from StressGen (Vancouver, BC) and resuspended in a buffer containing 20mM Tris-HCL pH 7.5, 100mM NaCl, 0.1mM EDTA, and 1mM DTT. Granzyme B ((MES pH 6.0 with 400mM NaCl) (32,000 mOD min^{-1}/ml, Assayed with Ellman's to detect the HSBL leaving group from 90 μM Boc-Ala-Ala-Asp-Sbzl in 0.1M HEPES pH 7.5 with 0.5M NaCl at 410nm) was a kind gift from Dr. Dorothy Hudig, University of Nevada, Reno.

Clonogenic survival. Colony-forming assays were performed as previously described (Carper et al.,, 1991). Briefly, following a heat shock all treated cells were harvested, counted with a Coulter counter and then incubated at various cell densities in supplemented media lacking G418 sulfate for 9-12 days to allow for colony growth. Colonies that grew from the surviving cells were stained with crystal violet and counted (>50 cells /colony). The percentage survival was calculated as 100 x (number of colonies formed/number of experimental cells plated)/(number of control colonies formed/number of control cells plated).

Caspase 3 Time Course. DC4 and DB46 cells were induced to undergo apoptosis with continuous exposure to 10mM NaB and assayed at the indicated times. After 0, 2, 4, and 6 hours, 5 x 10^6 cells were washed with phosphate buffer saline (PBS) and centrifuged. Lysis buffer was added at a ratio of 1x10^6cells/ 25μl buffer and the cells were incubated on ice for ten minutes. The cell suspensions were then centrifuged at 14,000 x g for fifteen minutes at 4^0C.

Fifty microliters of lysate was added to 50µl of assay buffer in a 96-well plate. Five microliters of the substrate (Asp-Glu-Val-Asp-pNA) were added to each well. Following incubation at 37°C for one hour, the absorbance at 510 nm was measured using a microplate reader (Dynatech MR5000). Results are presented as relative caspase 3 activity. Lysis buffer, assay buffer and substrate were used from a caspase 3 assay kit (Clone Tech.).

Caspase 9 Time Course. DC4 and DB46 cells were induced to undergo apoptosis at the indicated times with 10mM NaB. Five million cells were washed with PBS and then resuspended at a ratio of $1x10^6$/ 25µl of lysis buffer. Cells were incubated on ice for ten minutes and then centrifuged at 14,000 x g for fifteen minutes at 4°C. Fifty microliters of lysate were added to 50µl of assay buffer and then 5µl of caspase 9 substrate were added (Leu-Glu-His-Asp-pNA). Following an incubation at 37°C for one hour, the absorbance was read at 510nm on a microplate reader (Dynatech MR 5000). Results are presented as relative caspase 9 activity. The lysis buffer, assay buffer and substrate were obtained from Medical and Biological Laboratories Co. LTD.

Caspase 3, 8 and 9 Activity. Caspase 3 assay (Calbiochem) instructions were followed. Briefly, equimolar concentrations of recombinant caspase 3 and HSP 27 were added to wells in a 96 well plate, diluted to a volume of 90µl, and incubated at room temperature for ten minutes. Ten

microliters of the substrate (DEVD-pNA) was added to wells and the plate read every minute for 45 minutes at 510nm on a microplate reader (Dynatech MR 5000). Slopes of each line were compared and data reported as relative percent caspase activity.

Caspase 8 assay instructions were followed (Calbiochem). Briefly, equal molar concentrations of recombine caspase 8 and HSP 27 were added to wells in a 96 well plate and diluted to a volume of 90µl, and incubated at room temperature for ten minutes. Ten microliters of the substrate (IETD-pNA) was added to the wells and the plate read every minute for 45 minutes at 510nm on a microplate reader (Dynatech MR 5000). Slopes of each line were compared and data is presented as relative percent caspase activity.

Caspase 9 assay instructions were followed (Alexis). Equal molar concentrations of recombine caspase 9 and HSP 27 were added to wells in a 96 well plate and brought up to a volume of 90µl, and incubated at room temperature for ten minutes. Ten microliters of the substrate (LEHD-pNA) was added to the wells and the plate was analyzed every minute for 45 minutes at 510nm. Slopes of each line were compared and data is relative percent caspase activity

Immunoprecipitations. DC4 cell extracts were prepared as follows: 5×10^6 cells were washed in PBS and incubated in 600µl cytochrome c buffer (220mM mannitol, 68mM sucrose, 50mM PIPES-KOH, pH 7.4, 50mM KCl, 5mM EGTA, 2mM $MgCl_2$, 1mM dithiothreitol (DTT) and 1

pill of protease inhibitors (Complete Cocktail, Boehringer Mannheim)(Bossy-Wetzel, et al., 1998) for thirty minutes at room temperature. Cells were then homogenized for 40 strokes using a dounce homogenizer and a B pestle. Cells were then centrifuged for fifteen minutes at 14,000 x g. To the supernatant extract, 5µl of HSP 27 (1µg/µl) and 5µl cytochrome c (1µg/µl) were added. The lysate was allowed to incubate for ten minutes at room temperature. The lysate was then divided into two samples. To one sample HSP 27 antibody (StressGen) (1:200) was added and cytochrome c antibody (Pharmingen) (1:200) was added to the other sample. Samples were incubated on a lab quake for sixty minutes at 4°C. To all samples 20µl of A/G protein and agrose was added and samples were incubated for forty-five minutes on a lab quake at 4°C. Samples were centrifuged at 14,000 x g for 15 minutes, the supernatant discarded, and the pellet resuspended in denaturing/reducing sample buffer (125mM Tris pH 7.4, 4% SDS, 10% glycerol, .005% Bromophenol Blue, 2% B-mercaptoethanol). Samples were electrophoresed on a 10.0% separating gel at 100V for 45 minutes. The samples were then blotted (Bio Rad) for eighty minutes at 100mV onto nitrocellulose paper. The nitrocellulose paper was transferred into blocking solution (0.5% Blotto, 0.05% BSA in 1X TrisBorateSaline-Tween20 (TBST)) for 24 hours at 4°C. The primary antibodies (1:1000) were dissolved in 1X TBST, added to each paper, and allowed to shake for sixty minutes. The blots were then washed three times in 1X TBST for ten minutes. The secondary goat anti mouse antibody (Pharmingen) was dissolved in 1X TBST (1:2500) and added to the membranes. This was then shaken for one hour at room temperature, and three more washes with 1X TBST were performed. Finally, 1ml of the

chemilumensence substrate was added to each membrane and exposed to x ray film for thirty seconds. The results are shown on the western blots.

Cell Free Extracts. Five million DC4 cells were harvested and washed in PBS. Six hundred microliters of cytochrome c buffer were added to 2.5 x 10^6 cells (220mM mannitol, 68mM sucrose, 50mM PIPES-KOH, pH 7.4, 50mM KCl, 5mM EGTA, 2mM MgCl2, 1mM dithiothreitol (DTT) with 1 of the pill protease inhibitors (Complete Cocktail, Boehringer Mannheim))(Bossy-Wetzel, et al., 1998). Following incubation for ten minutes on ice the cells were homogenized with a dounce homogenizer with a B pestle for forty strokes. The cell lysate was then aliquoted into two 1.5ml microfuge tubes and centrifuged at 14,000 x g for fifteen minutes at 4^0C. Cell lysate was used with the caspase 3 assay kit (Calbiochem). The volume of cell lysate (75µl) and substrate (10µl) remained constant and the amounts of 1mg/ml cytochrome c (Sigma) and assay buffer was varied for a final volume of 100µl. Various concentrations of HSP 27 (StressGen) and cytochrome c (Sigma) were used in the experiments. Slopes of the line for thirty minutes were compared to the control and data is expressed as relative caspase 3 activity.

Granzyme B. Five million DC4 cells were washed in PBS, and 600µl of cytochrome c buffer/2.5 x 10^6 cells was added. Following incubation on ice for ten minutes, the cells were homogenized

with a dounce homogenizer for forty strokes with a B pestal. The cell lysate was then put into two 1.5ml microfuge tubes and centrifuged at 14,000 x g for fifteen minutes at 4^0C. Cell lysate was used with the caspase 3 assay kit as previously described (Calbiochem). The amount of lysate and substrate remained the same with additions of cytochrome c or granzyme B (either alone or in combination with equal molar amounts of HSP 27) being made 10 minutes prior to the assay. Assay buffer was used to bring the total volume up to 100µl. The kinetic assay was performed for thirty minutes. The slopes of the lines were compared to the control and data is reported as relative caspase activity.

Statistical Analysis. Results are reported as standard error of the means ± S.E.M. Statistical analysis was performed with a statistical software package (student version of Minitab). Statistical significance was determined by one-way ANOVA for repeated measurements followed by a student t-test.

Acknowledgements:

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Table 1 Effect of Hsp27 on Caspase Activity

% Caspase Activity

Addition	Caspase 3	Caspase 8	Caspase 9
None	100 +/- 10	100 +/- 6	100 +/- 8
Hsp27	105 +/- 12	100 +/- 10	103 +/- 9

Figure 1. Hsp27 inhibits cell death. Percent survival of DB46 (■) and DC4 cells (□) 14 days after 1 hr exposure to cyclohexamide (CHX), sodium butyrate (NaB) or vitamin E succinate (VES). Error bars represent standard error of means. Results are from one experiment done in triplicate. (*: p=0.05)

Figure 2. Hsp27 inhibits caspase 3 activation. Time course for caspase 3 activity in DB46 (●) and DC4 (○) cell lines at 0, 2, 4, 6, and 8 hours following induction of apoptosis by 10 mM sodium butyrate (NaB). Error bars represent standard error of the means, results are reported as the average of three experiments.

Figure 3. Hsp27 inhibits caspase 9 activation. Time course for caspase 9 activity in DB46 (●) and DC4 (○) cell lines at 0, 15, 30, and 45 minutes after treatment with 10 mM sodium butyrate. Error bars represent standard error of the means.

Figure 4. Hsp27 and cytochrome c complex formation. Western blot of Immunoprecipitations showing direct interaction between hsp27 and cytochrome c in vitro. Figure 4A is a western blot probed with an antihsp27 antibody. Figure 4B is a western blot probed with an anticytochrome c antibody. Additions are indicated with a +. Cell extracts were from the DC4 cell line.

Figure 5. Relative caspase 3 activity in the presence of various amounts of cytochrome c in DC4 cell lysate. Open bar indicates no addition of cytochrome c and filled bars indicate the addition of cytochrome c. Error bars represents standard error of the means, for 1 to 10 micrograms of cytochrome c the standard error was so low that error bars are too small to be seen.

Figure 6. Hsp27 inhibits cytochrome c activation of caspase 3 in cell free extracts. Relative caspase 3 activity in the presence of various concentrations of cytochrome c (in micrograms) (filled bars) with and without the presence of 5 micrograms of Hsp27 (+27) in DC4 cell lysate. Open bar indicates no addition of cytochrome c.

Figure 7. Granzyme B can overcome Hsp27 inhibition of caspase activation. Relative caspase 3 activity in the presence of 2 micrograms of cytochrome c (■) or 1 microgram of granzyme B (□) with and without the addition of equal molar amounts of hsp27 (+27) in DC4 cell lysate. Open bar indicates no addition of cytochrome c. Error bars represent standard error of the means and * indicates a $p = 0.05$ compared to the control.

Figure 8. Hsp27 protects breast cancer cells from cytochrome c cytotoxicity. Clonogenic survival of DB46 (●) and DC4 (○) cell lines at various concentrations of cytochrome c. Error bars represent standard error of the means.

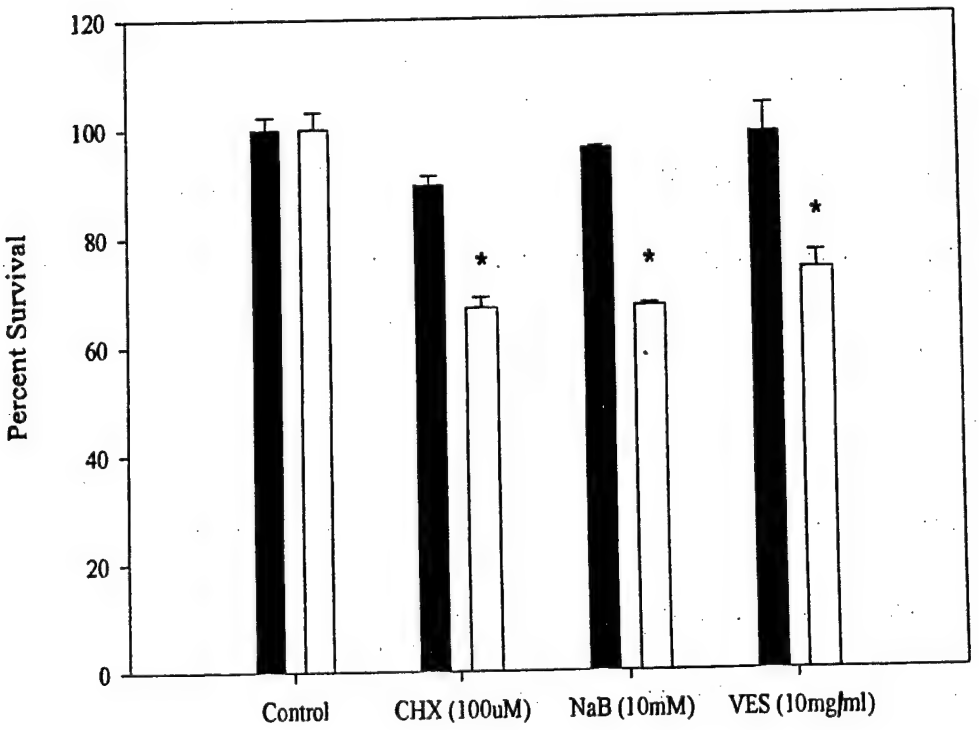

Percent Survival
120
100
80
60
40
20
0
*
*
*
Control
CHX (100uM)
NaB (10mM)
VES (10mg/ml)

Fig 1

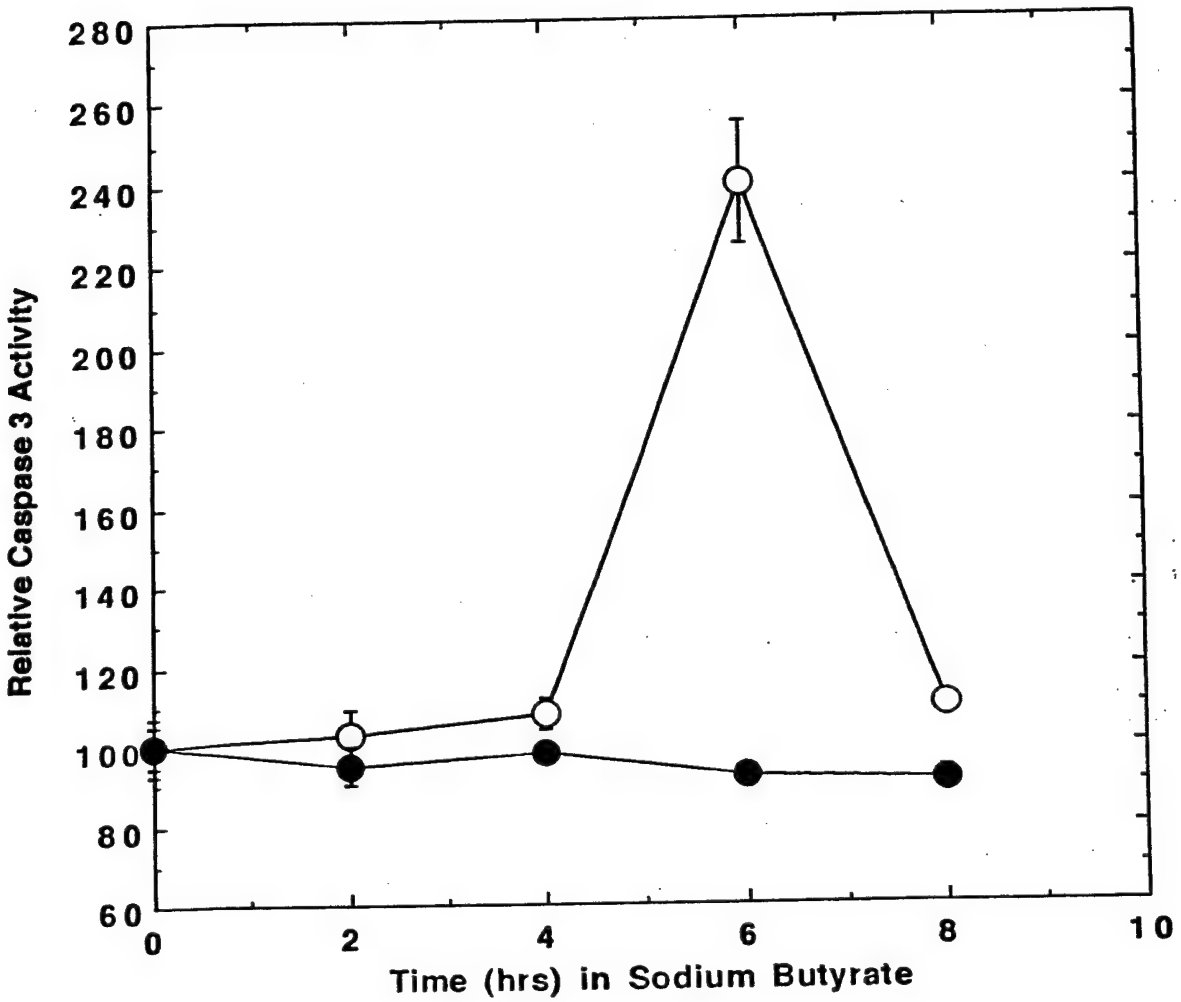

Relative Caspase 3 Activity
280
260
240
220
200
180
160
140
120
100
80
60
0
2
4
6
8
10
Time (hrs) in Sodium Butyrate

Fig 2

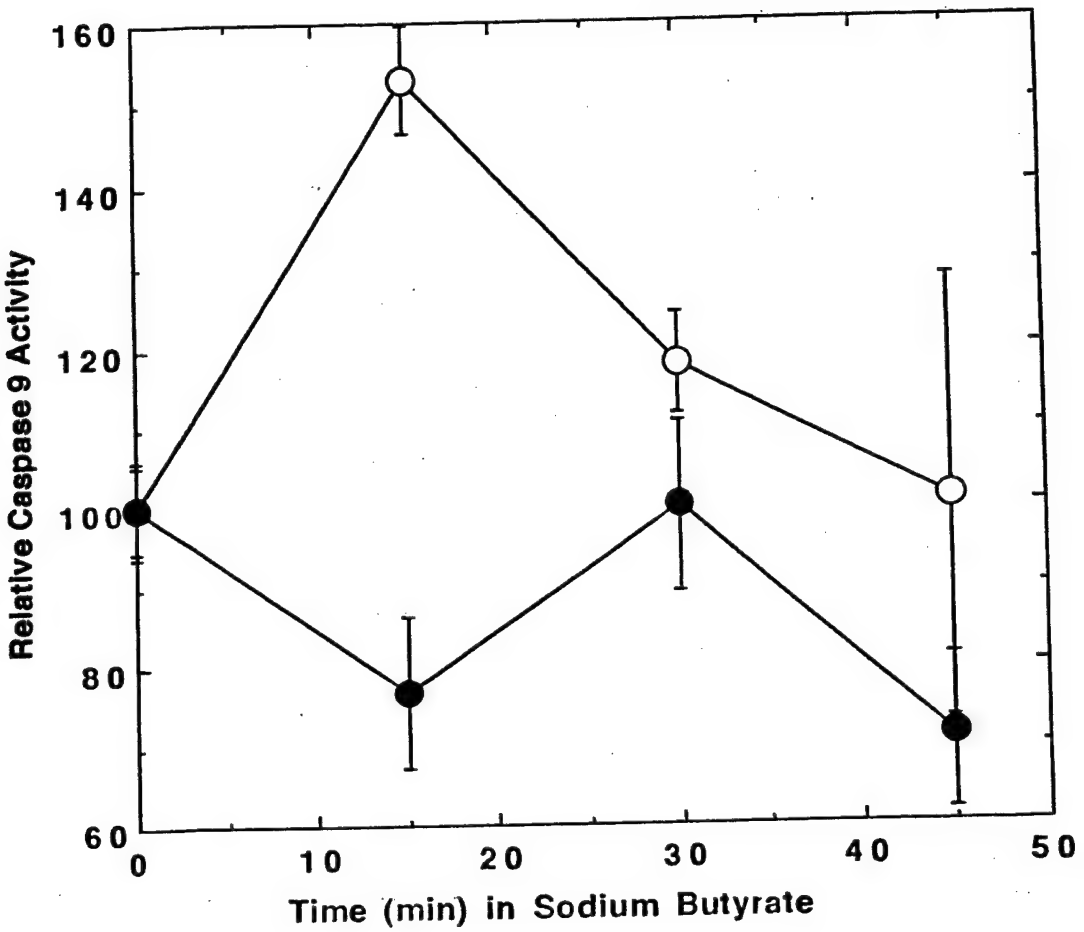
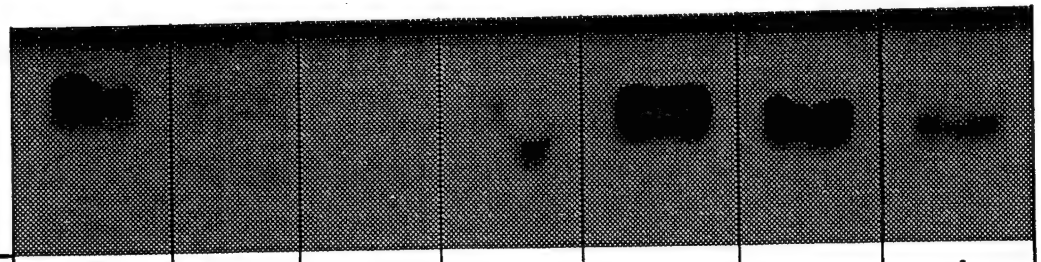

160
140
120
100
80
60
Relative Caspase 9 Activity
0
10
20
30
40
50
Time (min) in Sodium Butyrate

Fig 3

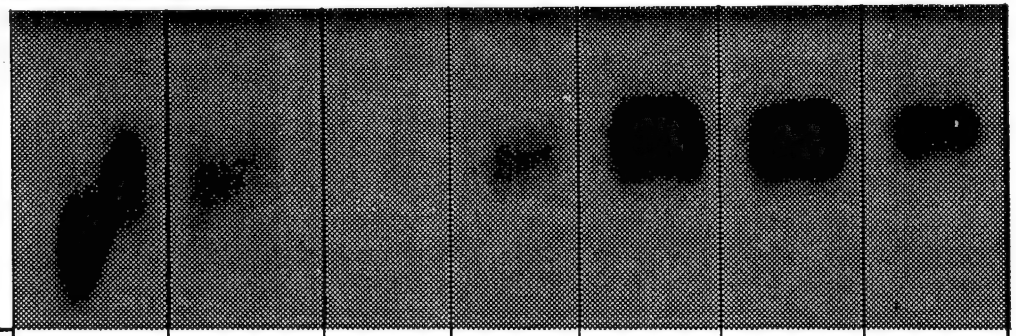
Hsp27 →



A

Cell extracts				+	+	+	+
Cyto. c						+	
Hsp27	+	+	+			+	+
Cyto. c Ab			+	+		+	+
Hsp27 Ab		+			+		

Cyto. c →



B

Cell extracts				+	+	+	+
Cyto. c	+	+	+			+	+
Hsp27						+	
Cyto. c Ab		+			+		
Hsp27 Ab			+	+		+	+

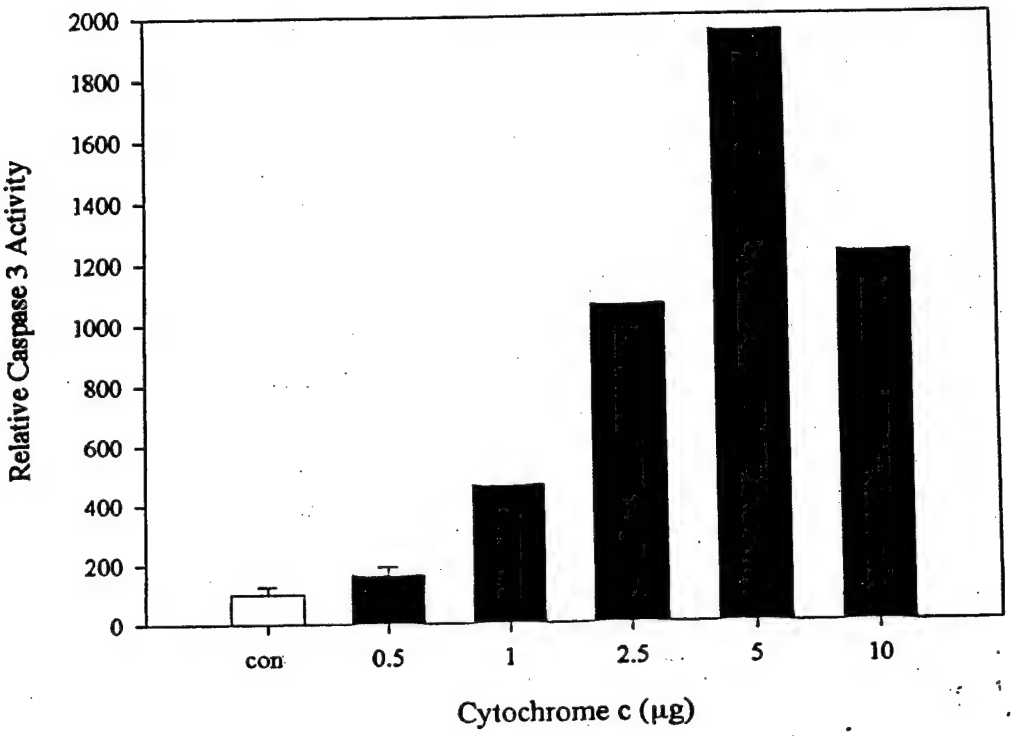

Relative Caspase 3 Activity
2000
1800
1600
1400
1200
1000
800
600
400
200
0
con
0.5
1
2.5
5
10
Cytochrome c (µg)

Fig 5

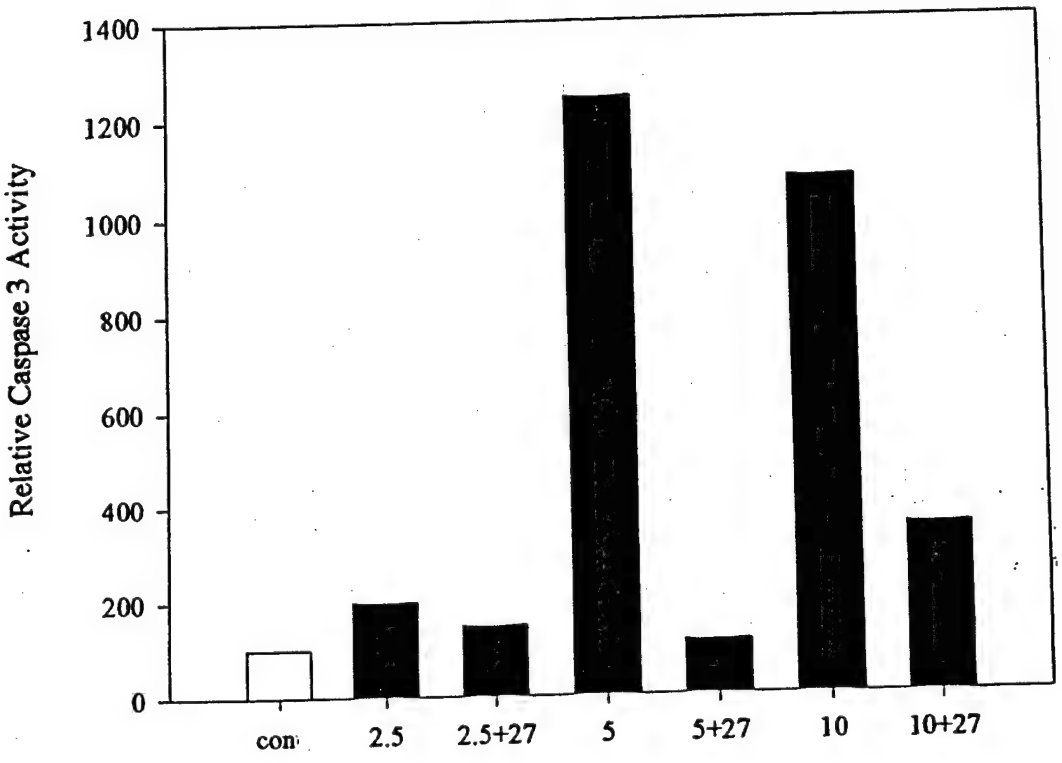

Relative Caspase 3 Activity
1400
1200
1000
800
600
400
200
0
con
2.5
2.5+27
5
5+27
10
10+27

Fig 6

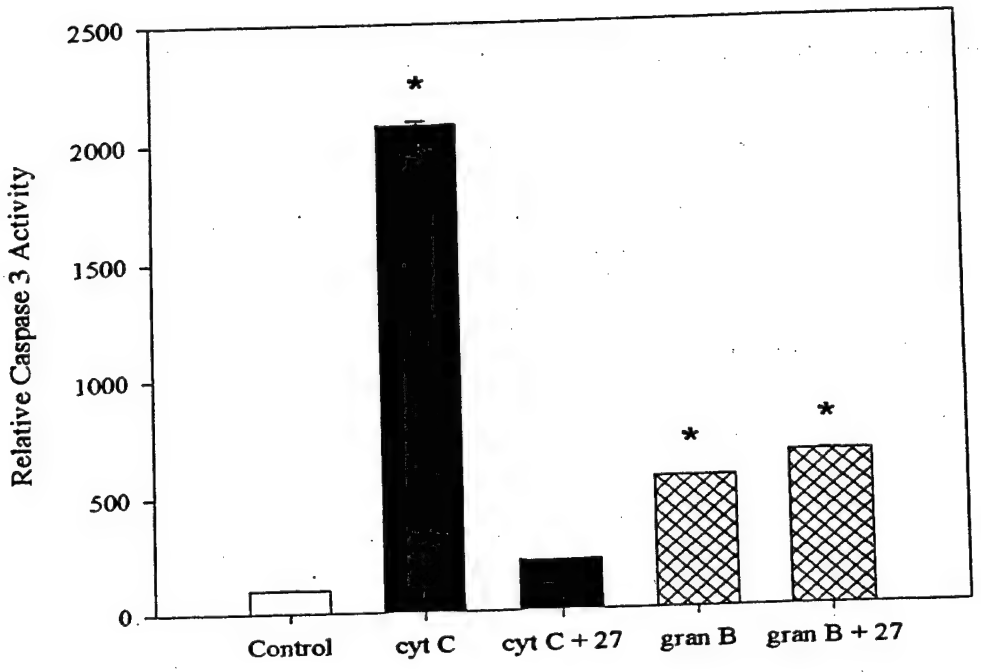

Relative Caspase 3 Activity
2500
2000
1500
1000
500
0
*
*
*
Control
cyt C
cyt C + 27
gran B
gran B + 27

Fig 7

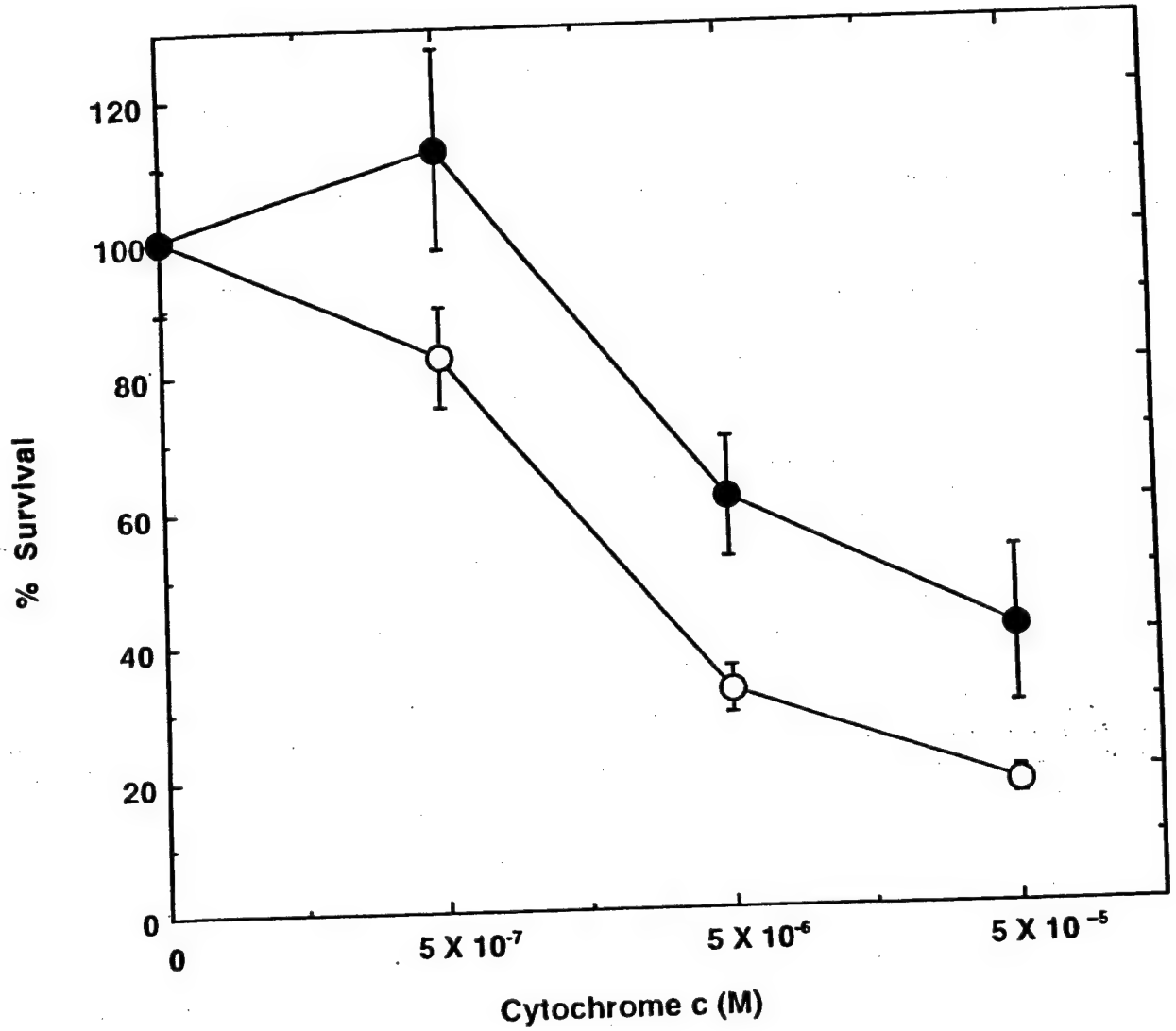

120
100
80
60
40
20
0
% Survival
0
5 X 10-7
5 X 10-6
5 X 10-5
Cytochrome c (M)

Fig 5

Appendix B

Caspase 9 Inhibition in the Caspase Cascade

APPENDIX B

Caspase 9 Inhibition in the Caspase Cascade

Materials and Methods

Cytochrome c Assays

Five million DC 4 cells (-HSP 27) were harvested and washed in PBS. Six hundred microliters of cytochrome c buffer were added to 2.5 x 10^6 cells (220mM mannitol, 68mM sucrose, 50mM PIPES-KOH, pH 7.4, 50mM KCl, 5mM EGTA, 2mM MgCl2, 1mM dithiothreitol (DTT) with 1 of the pill protease inhibitors (Complete Cocktail, Boehringer Mannheim))(36). Following incubation for ten minutes on ice the cells were homogenized with a dounce homogenizer with a B pestle for forty strokes. The cell lysate was then aliquoted into two 1.5ml microfuge tubes and centrifuged at 14,000 x g for fifteen minutes at 4^0C (36). Cell lysate was used with the caspase 3 assay kit (Calbiochem). The volume of cell lysate (75µl) and substrate (10µl) remained constant and the amounts of 1mg/ml cytochrome c (Sigma) and assay buffer was varied for a final volume of 100µl. Various concentrations of HSP 27 (StressGen) and cytochrome c (Sigma) were used in the experiments. Slopes of the line for thirty minutes were compared to the control and data is expressed as relative caspase 3 activity.

A caspase 9 inhibitor (Leu-Glu-His-Asp-CHO) (Alexis) was used in two concentrations (5µl and 10µl) to determine if caspase 9 was being activated in the caspase cascade. The above procedure was used and caspase 9 inhibitor was added prior to the start of the assay. The assay was conducted for thirty minutes and slopes of the lines were compared to the control.

Results

Caspase 9 is in the Caspase Cascade

Caspase 9 is activated during the addition of cytochrome c. This was demonstrated by adding a caspase 9 inhibitor and measuring caspase 3 activity (figure 10, next page). This demonstrated that if caspase 9 is inhibited, then caspase 3 activity will be reduced. With the presence of cytochrome c there is an increase in caspase 3 activity which was reduced by the caspase 9 inhibitor. When the inhibitor is added it lowers the caspase 3 activation by about 65%. Since the caspase 9 inhibitor did not completely inhibit the caspase 9 activation other caspases might also be activating caspase 3. However, it should be noted that the caspase 9 inhibitor was not 100% effective against activated caspase 9. This is due to the fact that the inhibitor is a competitive inhibitor and is concentration dependent. A conclusion can be made that a majority of the caspase 3 activation results from active caspase 9.

Reference:

36. Bossy-Wetzel, E., Newmeyer, D. D., and Green, D. R. (1998) EMBO J. **17** 37-49

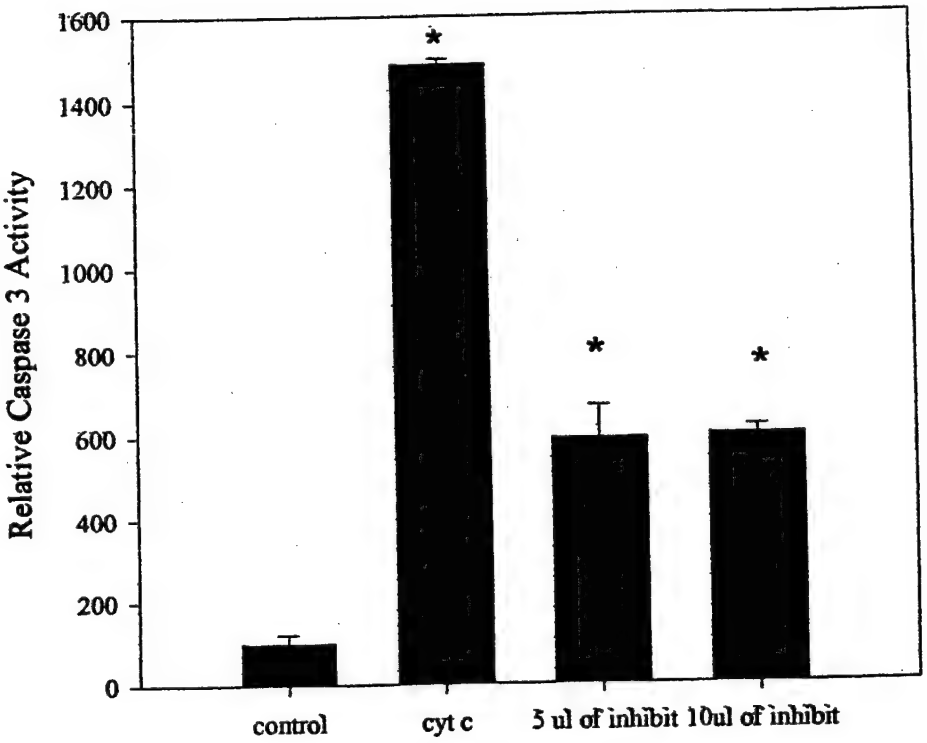


Figure 10 Effects of caspase 9 inhibitor on caspase 3 activation in cell free lysate. Relative caspase 3 activity in the presence of various amounts of caspase 9 inhibitor. Error bars represent standard error of the means. (* : p=0.05 compared to control)

Appendix C

Cytochrome c Can Overcome HSP27 Inhibition of Apoptosis in Human Breast Cancer Cells

DRAFT

Cytochrome c Can Overcome HSP27 Inhibition of Apoptosis in Human Breast Cancer Cells

by

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Bachelor of Science
University of Nevada, Las Vegas
2000

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University of Nevada, Las Vegas
2003

DRAFT DRAFT

A thesis submitted in partial fulfillment
of the requirements for the

Master of Science in Biochemistry Degree
Chemistry Department
College of Sciences

Graduate College
University of Nevada, Las Vegas
July 2003

ABSTRACT

Cytochrome c Can Overcome HSP27 Inhibition of Apoptosis in Human Breast Cancer Cells

by

Yvonne C. Giraud

Dr. Stephen W. Carper, Examination Committee Chair
Associate Professor of Chemistry
University of Nevada, Las Vegas

The human breast cancer cell lines DC4, DB46, MCF-7, MDA-MD-435, the human colon cancer cell line RKO and the normal human lung fibroblast cell line HFL1were used in experiments. DC4, DB46, MCF-7, MDA-MD-435 were treated for one hour with exogenously added cytochrome c at $5x10^{-7}$M, $5x10^{-6}$M and $5x10^{-5}$M. A dose response to cytochrome c exposure was seen in the breast cancer cell lines. RKO and HFL1 were exposed to cytochrome c at $5.6x10^{-8}$M, $5.6x10^{-7}$M and $5.6x10^{-6}$M. RKO showed no sensitivity to cytochrome c exposure at any of the concentrations and HFL1 showed sensitivity only at the highest concentration. FAD, NAD^+, Q_{10} and ATP were incubated with cells at $1x10^{-9}$M, $1x10^{-8}$M and $1x10^{-7}$M. It was found that NAD^+ was most effective, followed by FAD, in cell death induction at much lower concentrations than cytochrome c. ATP had some effect and Q_{10} was the least effective in cell death induction.

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CHAPTER 1

INTRODUCTION

Heat Shock Proteins

The name heat shock proteins (HSPs) was coined in 1962 as Ritossa discovered that a certain group of proteins became expressed in *Drosophila* larvae following exposure to temperatures that were above those allowing optimal growth (1). Through further research by Tissières it was soon realized that high temperatures were not the only method of induction (2,3). Studies showed that metals, amino acid analogs and anoxia were able elicit similar responses; hence, HSPs are also referred to as stress proteins. HSPs are divided into five major families according to their molecular weight: HSP100, 90, 70, 60 and the small HSP (sHSP)/α-crystallins (3). sHSP are evolutionary related to α-crystallins which is known to be a major structural protein of the vertebrate eye lens. A common characteristic of the α-crystallins/sHSP family is a conserved homologous sequence of 90-100 residues (4). HSPs are found in all organisms from bacteria to humans and are among the most conserved proteins. The synthesis of HSPs allows an organism to gain tolerance to insult. An insult constitutes elevated temperature or stress. It was found that HSPs are synthesized in stressed cells as well as in unstressed cells, leading to the understanding that HSPs fulfilled another function. The major role for large HSPs, which are HSP100, 90, 70 and 60, appears to be molecular chaperones (3,4). Molecular chaperones have been defined as proteins that bind to and stabilize an otherwise unstable conformer of another protein. The controlled binding and release of the client protein determines its correct fate *in vivo*, which may be folding, oligomeric assembly, transport to a particular subcellular compartment or controlled switching between active versus inactive conformations (2). HSP100/Clp proteins protect cells under stress conditions. They function as chaperones, solubilize protein aggregates

in an ATP-dependent manner and lead either to renaturation or proteolytic degradation of individual proteins (3). HSP90 under normal conditions is an abundant, essential, cytosolic protein that in a complex with other proteins engages steroid receptors to possibly maintain them in an inert, folded state allowing for the binding with and reaction to the correct steroid signal (3). Stressed cells show a marked increase of HSP90 (3). HSP70 is a constitutive and stress-inducible protein. It binds to nascent polypeptide chains on ribosomes, maintains the membrane translocation-competent state of the precursor proteins, assists protein transport into mitochondria and the endoplasmic reticulum as well as protects proteins under stress (3). HSP60 belongs to the chaperonin family, which includes GroEL, from bacteria among others, which is a group of proteins with a distinct ring-shaped, or toroid (double donut) quartenary structure. The chaperonins appear to mediate native folding of proteins in an ATP-dependent manner, which seems to proceed through cooperation of HSP70 (3). It is suggested that in this way large HSPs contribute to the maintenance and restoration of cellular homeostasis during and after stress (3). sHSP/α-crystallins form a diverse family of proteins. They are between 15-30 kDa in size, can be induced by mild heat shock or other forms of stress, all have a tendency to aggregate and are involved in protein-protein interactions (4). The focus will now turn to the small heat shock protein 27, HSP27, and its role in programmed cell death (apoptosis).

Heat Shock Protein 27

HSP27 belongs to the family of sHSP/α-crystallins. This small heat shock protein is constitutively expressed in many cell types and tissues at specific stages of development and differentiation (5). A variety of stimuli including elevated temperature, oxidative stress, staurosporine, Fas ligand and cytotoxic drugs induce HSP27 protection against apoptosis (5). Intracellular localization, level of oligomerization and phosophorylation status seem to determine its biological reactivity (2).

A commonality found between α-crystallin and HSP27 is the phosphorylation at specific serine residues. The mammalian HSP27 has three major phosphorylation sites, which are at

Ser-15, Ser-78 and Ser-82 (4,9). The monomeric, non-phosphorylated HSP27 has been shown to be the only form that inhibits actin polymerization *in vitro* (2). Elevated levels of HSP27, particularly phosphorylated isoforms, have been associated with increased resistance of human breast cancer cells to doxorubicin (4). Rapid stress-induced phosphorylation is induced by a p38 MAP (mitogen-activated protein) kinase cascade with subsequent activation of MAPKAP (mitogen-activated protein kinase-acticated protein) kinases 2 and 3, which directly phosphorylate mammalian sHSPs at several distinct sites (11). Increased phosphorylation of mammalian sHSPs occurs in response to a number of mitogenic stimuli including serum, growth factors and tumor promoters. Calcium ionophores and chelators, cadmium, arsenite, cyclohexamide and phorbol esters are known to induce phosphorylation at normal growing temperature (4). Phosphorylated HSP27 forms smaller rod-like oligomers, which may be tetrameric while nonphoshorylated HSP27 exists in larger complexes made up of about 24 subunits (6 tetramers). Dissociation of large oligomers to tetramers as a result of phosphorylation or of mutation of all three serine residues to aspartate has been shown to lead to a significant decrease in chaperone activity (11).

HSP27 has been shown to correlate with the oncogenic status of cells and plays a role in their tumerogenicity (5). HSP27 is overexpressed in several human breast carcinomas and correlates well with the level of estrogen receptors. (6). HSP27 is an estrogen responsive protein (4,6,7). High levels of HSP27 correlated with a shorter disease-free survival period in node-negative patients but showed prolonged survival from first occurrence (6). Elevated levels of HSP27 in advanced cancers have been shown to be indicative of long survival. The postulation is that there is a link with hormone response (6). Elevated levels of HSP27 may be in response to environmental stresses or exposure to estrogen (7). Treatment with 17 β-estradiol was able to educe HSP27 expression in human breast cancer cell lines (7). Breast cancer cell lines overexpressing HSP27 showed an increase in cell survival when treated with doxorubicin or heat (7).

The estrogen receptor, in absence of hormone, is not bound to its respective hormone

response element (HRE). The hormone receptors are present within the nucleus bound to a complex consisting of two molecules of HSP90, one molecule of HSP70 and other proteins (8). Upon hormone binding, the receptors are released from the hsp complex (8). This homodimer has a greatly increased affinity for binding to HREs in DNA. The HREs are usually found several hundred base pairs upstream of target genes (8) The estrogen homodimer can bind to the HRE in the promoter region of the HSP27 gene and transcription of the HSP27 gene is initiated. Thus, when estrogen is present the HSP27 protein can become expressed in elevated levels (9).

HSP27 has been shown to block apoptosis induced by Fas (death cell surface receptor belonging to the tumor necrosis factor (TNF) superfamily of surface receptors) by binding to DAXX (10). DAXX is an adapter protein that is normally associated with the nuclear substructures named ND-10 or PML oncogenic domain (POD) (10,17). This adapter protein can bind to the Fas receptor, mediate Fas-induced apoptosis and activate apoptosis signal-regulated kinase 1 (Ask 1) (10). Activation of this kinase can then lead to activate the stress-activated protein kinase 1-Jun N-terminal kinase (SAPK1/JNK) (10). Thus, the binding of HSP27 to DAXX prevents its interaction with Fas and Ask 1 (10).

In neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, amyotropic lateral sclerosis and progressive supranuclear palsy the production of reactive oxygen species (ROS) has been implicated in the pathogensis. Unphosphorylated, large oligomers of HSP27 protect the cell against ROS and play a role in confluent-dependent resistance (12,13). It is suggested that the protection conferred against oxidative stress is independent of its ability to bind cytochrome c, which is an electron carrier in the mitochondrial electron transport chain (12). HSP27 has been shown to play a significant role in inhibiting apoptosis by binding to cytochrome c (5,14,15). It appears that both, *in vitro* chaperone propterties and the ability to protect against oxidative stress depend on the existence of large oligomers of HSP27 meaning that *in vivo* chaperoning by HSP27 is the basis for its protective activity at the cellular level (11). The conserved ability of sHSPs to protect against oxidative stress appears to depend on their ability to raise the intracellular concentration of glutathione and perhaps to maintain this redox modulator in

its reduced form during oxidative stress (1,11).

Apoptosis

Apoptosis plays a pivotal role in maintaining the balance between cell proliferation, regulation of healthy development, and protection against disease (17). It is involved in fundamental processes such as the regulation of immune cell maturation, target cell death induction by cytotoxic T lymphocytes and natural killer (NK) cells as well as the response of hormone-growth-factor-dependent tissues to the addition of withdrawal of ligands (17). Apoptosis is a morphologically distinct form of cell death designed to rapidly remove unwanted and potentially dangerous cells. The inappropriate regulation of apoptosis is associated with a variety of diseases, including cancer, AIDS, neurodegenrative diseases and ischemic stroke (18).

Cells undergoing apoptosis are characterized by distinct morphological alterations including cell shrinkage, nuclear condensation, and the blebbing of apoptotic vesicles containing intracellular components. Those vesicles are specifically recognized and engulfed by phagocytic cells; hence, an inflammatory response is not elicited because intracellular contents are not released into the extracellular environment (17). Apoptosis is fundamentally different from necrosis. Necrosis is the result of acute cellular disfunction due to extreme trauma or injury to the cell. This passive and disruptive process results in rapid loss of ion flux, leading to the uptake of water, which gives rise to cell and organelle swelling ending in cytolysis. The release of the cell contents into surrounding tissue solicits a local inflammatory response (2).

Characteristic biochemical alterations that occur during apoptosis include DNA cleavage between nucleosomes to form fragments that are multiples of approximately 180 basepairs and /or into 50-to 300-kb fragments, the reorientation of phosphatidylserine form the cytoplasmic to the extracellular face of the plasme membrane and the loss of the mitochondrial membrane potential (17).

Cells are exceptionally sensitive to small changes in their environment and have to be able to adapt quickly to new conditions. Many times a rapid response is facilitated by the constitutive

expression of key components of signal transduction or effector pathways (17). Many cells are able to undergo apoptosis in the presence of inhibitors of macromolecular synthesis, leading to the indication that the distal components of the apoptotic pathway are constitutively expressed. It has been shown that such inhibitors are capable to induce apoptosis in some cell lines independent of additional stimulus thus it appears that the constitutively expressed effectors of apoptosis are normally inhibited by suppressive molecules (17). The apoptotic pathways were elucidated from studies performed in the nematode *Caenorhabditis elegans* (17,19-26). It was discovered that the gene *ced*-3 shares homology with a family of cysteine proteases known as caspases in higher animals (17). The term caspase refers to the enzymatic specificity: *Cys* protease cleaving after *Asp* residues (17,22,23,26). The human *ced*-4 homologue is *apaf*-1 (apoptotic protease-activating factor-1).

More than 14 caspases have been identified adding to the complexity to the pathways controlling apoptosis in higher animals (17,19,20). All of them have similar amino acid sequences and substrate specificities, all cleave after an apartic acid residue and all recognize a motif of at least four amino acids proximal to the cleavage site; yet, other structural elements must be required for substrate recognition since not all target proteins with a particular motif are cleaved (17).

Caspases are synthesized as proenzymes (zymogens), consist of three domains (N-terminal prodomain together with large (~p20) and small (~p10) subunits), remain inactive in most healthy cells and require proteolytic cleavage to be active (14,17-23). Activation occurs as the single-chain procaspases are cleaved at specific aspartic acid residues to remove an inhibitory N-terminal pro-domain and the cleavage at a specific recognition site between the large and small subunits to generate two distinct subunits compared to most non-caspase proteases, which are activated by removal of the inhibitory domain (18,19). Two subunits associate to form a heterodimer (17,18). Mature (activated) caspases form a tetramer, which are two heterodimers bound together, consisting of two large and two small subunits (21).

Acitvated caspases exhibit considerable substrate specificity, particularly in relation to effector

capases (19). Capases that are involved in apoptosis are divided into two groups: group III, which is involved in the initiation of the apoptotic signal (initiator or apical caspases) and group II, which is involved in the disassembly of the cell (effector or down-stream executioner caspases) (14,17,19). Initiator caspases preferentially cleave at recognition sites that consist of the following four amino acid sequences YexD (Y→I, L, V and x→any amino acid) whereas effector caspases prefer DexD sequences (each capital letter represents an amino acid, listed in Table 1) (19). As mentioned earlier, all caspases exist in cells as inactive zymogens that have to be activated in order to be able to induce apoptosis. Effector caspases (e.g. caspase-3 or -7) are activated by initiator caspases (such as caspase-9) via cleavage at specific internal aspartate residues separating the large and small subunits (17,22,23,26).

There are two pathways that can induce the activation of initiator caspases. One pathway leading to caspase activation is initiated by engaging cell surface death receptors with their specific ligands and the other is known as mitochondrion-dependent apoptosis (17,26-28). An example of death receptor apoptosis will be briefly discussed and the focus will then be turned to mitochondrion-dependent apoptosis.

In the apoptotic pathway of cell surface death receptors (figure 1, page 19), a number of cytokines that belong to the tumor necrosis factor receptor (TNFR) superfamily (a family of transmembrane proteins) induce apoptosis (2,17,27,28). Mammalian death receptors include FAS/APO-1/CD95, TNFR1, DR-3/APO-3/WSL-1/TRAMP, and the TRAIL receptors DR4/TRAIL-R1, DR5/TRAIL R2/TRICK2/ KILLER (17,27,28). These death receptors consist of characteristic cysteine-rich extracellular repeats but lack substantial homology in their cytoplasmic regions except in the death domain (17,27,28). The death domain spans approximately 80 amino acids and is required for the death signal transduction (10,17). Fas and TNFR share a region of homology at the death domain at the cytoplasmic face (68 amino acids), which is required for apoptotic signaling by both Fas and TNFR (17). The activation ligands for these death receptors are structurally related molecules that belong to the TNF gene superfamily; hence, Fas/CD95 ligand (FasI) binds to Fas, TNF and lymphotoxin α bind to TNFR1, Apo 3 ligand (Apo3L) binds

to DR3 and APO2 ligand (Apo2L or TRAIL) binds to DR4 and DR5 (17,27,28). The Fas receptor is the only member that appears to act primarily in the apoptotic pathway. It is expressed in diverse tissues but is mainly involved in the modulation of the immune response (17). Binding of FasL to Fas receptor induces trimerization of Fas (18,27). A death domain-containing (DD-containing) adaptor molecule designated FADD/Mort1 (Fas-associating protein with death domain/Mort1), which contains a DD at its C-terminus, is then recruited to the cytoplasmic region of Fas where it binds to the DD of Fas, which is then called the ligand bound-death receptor complex (death-inducing signaling complex or DISC) (17,18,27). The N-terminus of FADD, termed the death effector domain (DED) is essential for recruiting the upstream initiator procaspase-8 and/or procaspase-10 (17,27,28). Procaspase-8 immediately undergoes autoactivation upon the recruitment to DISC and then activates procaspase-3, which is the procaspase that mitochondrial-dependent apoptosis and death receptor apoptosis pathways converge on (17).

Another protein that normally localizes to sub-nuclear structures known as ND-10 or PODs (PML-oncogenic domains) can associate with the Fas receptor (10,17). This protein is known as Daxx and its association with the Fas receptor activates the JNK apoptotic pathway (17).

Death receptor induce apoptosis is regulated by three distinct mechanisms (27,28). In the first mechanism the recruitment and/or activation of procaspase-8 at the DISC is prevented by a group of inhibitors belonging to a family of viral proteins, FADD-like ICE inhibitory proteins (vFLIPS) that contain two DEDs (27,28). The two DEDs compete with procaspases for binding to the DED of FADD; thus, preventing the recruitment of procaspases to the DISC (27,28). The mammalian homologue of vFLIP was identified and is known as Casper, I-FLICE, FLAME, CASH or MERIT (27). Two alternatively spliced forms of FLIP, FLIP-long and FLIP-short, exist (27). FLIP-long has a C-terminal that resembles caspase-8 but lacks protease activity because several conserved residues at the caspase active site are absent (27). Both isoforms of cellular FLIP bind to FADD, procaspase-8 and procaspase-10 by way of DED interactions (27).
A second mechanism is through the expression of decoy receptors for TRAIL (18,27). Decoy

receptors are closely related to the TRAIL receptors DR4 and DR5. These receptors lack the cytoplasmic domain (DcR1) or contain a cytoplasmic region with a truncated death domain (DcR2), thereby specifically inhibiting TRAIL-induced apoptosis by sequestering the TRAIL ligand away from the death receptors DR4 and DR5 (27).

The third mechanism involves viral protein crmA, a member of the serpin family that is a potent inhibitor or procaspase-8 (27). CrmA can inhibit the autoproteolytic activation of procaspase-8 as well as the ability of caspase-8 to cleave Bid, which leads to the release of cytochrome c from mitochondria resulting in the activation of downstream caspases (27).

In mitochondrion-dependent apoptosis (figure 2, page 20), mitochondria play a critical role in the induction of apoptosis by responding to a myriad of stimuli (29). In response to apoptotic stimuli these organelles release proteins into the cytosol that trigger caspase activation or other functions relevant to apoptosis, including cytochrome c (cyt-c), caspases, AIF (apoptosis-inducing factor), and SMAC (DIABLO) (second mitochondria-derived activator of caspases and murine homologue) (29,30).

A mitochondrion is a dynamic organelle comprised of an inner and outer membrane (31-33). Both membranes have differing levels of permeability and selectivity for cytoplasmic molecules and the organelle employs a diverse set of channels, pumps and transporters to move molecules across these membranes (31). Cytochrome c, which is nuclear encoded and upon synthesis transported to the inter-membrane space (IMS), is no exception (31). The addition of the heme group renders cytochrome c pro-apoptotic and serves to lock it in the IMS preventing its access to cytoplasmic *apaf*-1 (31). Apo-cytochrome c (lacking the heme) does not activate *apaf*-1, thus protecting the cell from cytochrome c while it is being synthesized, transported and contained within the mitochondria (31). It is a possibility that a threshold of cytochrome c is required to activate the apoptosome and therefore a cell may deal with the release of small amounts of cytochrome c in the cytoplasm during normal mitchondrial turnover (31).

In healthy cells, mitochondria maintain an electrochemical gradient ($\Delta\psi$) across their inner membrane that is created by pumping protons from the matrix to the IMS in conjunction with

electron transport through the respiratory chain (29,32,33). The electrochemical gradient is made up of ΔpH and ΔV_m components and is essentially caused by protons that are driven out of the matrix to the outer surface of the inner membrane (29,32,33). This proton pumping therefore creates a pH gradient and an electrical gradient across the inner membrane (32,33). The influx of protons back into the matrix is believed to be mediated mainly by the F_oF_1-ATPase/H^+ pump (29,32).

Depolarization of mitochondria and loss of the electrochemical gradient ($\Delta\psi$) is universally associated with apoptosis and cell death (29). A wide variety of apoptotic stimuli induce the releases of cytochrome c, which is a one-electron transfer agent involved in the electron transport in the respiratory chain, form mitochondria prior to membrane depolarization (29,32,33). These stimuli include growth factor or neurotrophin withdrawal, DNA-damaging drugs, radiation, protein kinase inhibitors such as staurosporine, elevations of pro-apoptotic Bcl-2 family proteins, a variety of agonists or antiagonists of the retinoid/steroid hormone family of nuclear receptors and cell surface death receptors (27,29). In response to these stimuli, mitochondrial depolarization typically occurs after caspase activation as a late event (29).

There are four distinct protein complexes that make up the mitochondrial respiratory chain (figure 3, page 21) (32,33). They are (I) NADH-coenzyme Q reductase, (II) succinate-coenzyme Q reductase, (III) coenzyme Q-cytochrome c reductase, and (IV) cytochrome c oxidase. Briefly, NADH donates electrons to Complex I, serving as a link between glycolysis, the TCA cycle, fatty acid oxidation and the electron transport chain (32,33). Complex I and II produce a common product, reduced coenzyme Q (UQH_2), which becomes the substrate for Complex III (32,33). Complex III oxidizes UQH_2 and reduces cytochrome c. Reduced cytochrome c then is the substrate for Complex IV and this is where molecular oxygen is reduced (32,33).

The apoptotic induction of cytochrome c from mitochondria into the cytosol marks the beginning of caspase activation. Cytochrome c, which is present in the concentration of between 0.5 and 5 mM in the mitochondrial intermembrane space, is released from the mitochondria within 5 minutes (31,34,35). Once in the cytosol, cytochrome c binds its cytosolic partner *apaf*-1 and

induces the oligomerization of *apaf*-1• cytchrome c complex in a dATP/ATP-dependent manner (33,34,38). *Apaf*-1 is a 130-kDa protein that consists of the following three domains: the N-terminal contains 85 amino acids that show homology with the prodomain of several caspases including caspase-1, -2 and -9 (19,20,23,24,27,38). This domain appears to function as the caspase recruitment domain (CARD) to bind caspases with a similar CARD (19,20,27). Caspase-9 is the only procaspase of all the CARD-carrying caspases to be activated by *apaf*-1 (27). After the CARD domain follows a stretch consisting of 310 amino acids that have 50% homology to the *C. elegans* death promoting domaind *ced*-4 (19,20,27,38). The most obvious conserved regions of this domain are the Walker's A and B boxes, which are believed to be necessary for nucleotide binding since mutations in this nucleotide-binding site abolish both *apaf*-1 and *ced*-4 function (19,20,27,38). The ATP synthase complex contains alpha- and beta-subunits, which ATP and ADP (32,37). The beta subunit contritbutes to the catalytic sites whereas the alpha subunit appears to be involved in the regulation of the ATP synthase activity (39). Amino acid sequences of alpha-and beta-subunits are weakly homlogous to each other and may have common functions in catalysis (39). Related sequences in both alpha- and beta-subunits as well as other enzymes that bind ATP and ADP, have helped to identify regions contributing to an adenine nucleotide binding fold in both ATP synthase subunits (39). The C-terminal half of *apaf*-1 contains 12-13 WD-40 repeats that mediate protein-protein interactions (19,20,21,27,38). WD-40 repeats are highly conserved repeating units found in all eukaryotes usually ending with residues WD (40). A truncated *apaf*-1, in which the WD-40 repeats have been removed, renders *apaf*-1 constitutively active *in vitro*, independent of dATP/ATP and cytochrome c (27,37,38). Conversely, activated caspase-9 cannot be released from *apaf*-1 when the WD-40 repeats are truncated, signifying that this domain normally fulfills dual functions to inhibit *apaf*-1 activity and to help release the activated caspase-9 (19,27,36).

The critical role of *apaf*-1 was confirmed by the analysis of *apaf*-1-deficient mice displaying abnormalities in several tissues, particularly the brain, as well as lack of developmental cell death (19,23,41). On the transcriptional level, the regulation of *apaf*-1has been implicated in several

important biological processes particularly in tumorigensis and during the development of the mammalian central nervous system (42). *Apaf*-1 has been described to be the core of the apoptosome and cytochrome c, which is essential for mitochondrial respiration and energy production, functions as a cofactor in the activation of caspases (41). All four components, cytochrome c, *apaf*-1, dATP/ATP and procaspase-9, form the apoptosome complex (15,20,21,27,36,38,41,42).

Cytochrome c is pivotal to the apoptosome assembly. Cell lines lacking cytochrome c and grown under conditions that mitigate the loss of this electron carrier, failed to form an apoptosome and failed to activate procaspase-9 in response to proapoptotic stimuli (38). Mitochondrion-dependent apoptosis requires activation of procaspase-9 to initiate the caspase cascade (16,36). This activation is dependent on the protein *apaf*-1, which has to be activated by cofactors cytochrome c and dATP (16). Cytochrome c binds *apaf*-1strongly in the absence of dATP; however, addition of dATP to the cytochrome c•*apaf*-1 complex results in further assembly producing a larger complex made up of 8-10 *apaf*-1 molecules along with associated cytochrome c molecules (16,22). The stoichiometry of the complex has been established to be 2cyt c•1*apaf*-1 and has been shown not to change with the addition of dATP (16). The role of dATP is to assist the formation of the apoptosome complex (16,22). *Apaf*-1 alone binds dATP poorly and in the presence of cytochrome c, nucleotide binding of *apaf*-1 increases about 10-fold, confirming the temporal sequence of binding events that take place (22,36). Furthermore, procaspase-9 increases dATP binding to *apaf*-1 indicating that the formation of the apoptosome is a synergistic event that stabilizes nucleotide binding (36). The stoichiometry of *apaf*-1 and procasase-9 binding is believed to be1:1 due to the observed CARD:CARD interactions (19,27). The assumption is that procaspase-9 autodigests at the *apaf*-1 and cleaves a neighboring procaspase-9 to form a tetramer and thereby forms the active form (19). Lastly, activated caspase-9 is released from the apoptosome and goes on to cleave and activate downstream caspases such as procaspases-3, -6, and -7 (27). Specific target substrates are then cleaved leading to apoptosis (15).

The apoptosome complex has been isolated and found to exist in two different sizes. There is one ~ 1.4 MDa complex that appears to be inactive and may be due to inappropriate oligomerization of the *apaf*-1 (19,20,26). Then there is an ~ 700 kDa complex that is capable of processing and activating effector caspases. It is this complex that has been found to be primarily assembled during apoptosis (19,20). The structure of the apoptosome has been hypothesized to be a wheel-like particle with seven spokes that radiate from a concave, central hub (38). The hub and bent spokes combine to give the apoptosome a puckered shape (38). The model suggests that cytochrome c interacts with WD-40 repeats that are contained in the spokes, places *apaf*-1 in the upper region of the hub and has dATP binding to the *ced*-4 homolgy region of the *apaf*-1 with procaspase-9 being tethered to the apoptosome via CARD:CARD interactions (38).

As has been described thus far, the molecular pathways that lead to the activation of caspases are tightly regulated. This regulation can be interfered with at the level of HSPs. The role of HSP27 in the inhibition of apoptosis has been investigated. It has been found that cytosolic HSP27, in human leukaemic cells, interacts with cytochrome c released from the mitochondrial IMS, therefore inhibiting apoptosome formation as well as caspase activation (5). It was shown that in a cell free system, HSP27, co-immunoprecipitated with cytochrome c and not with procaspase-9, *apaf*-1or procaspase-3 (5). Another study showed that heat shocked Jurkat cells had protection against apoptosis from HSP27 (15). By preventing the formation of the apoptosome, HSP27 aids in the formation of cancer.

Cancer

Cancer has two characteristics. One is uncontrolled cellular proliferation and the other is the failure of cells to undergo apoptosis (43). Normally, cells develop a specific morphology and function, which is known as differentiation (45). The process of differentiation causes normal cells to lose the ability to prolilferate (45). This process is kept in balance through the replacement of less-mature precursor cells also known as stem cells in renewal tissue (44).

Precursor cells then go on to differentiate and it is from these precursor cells that cancer most likely originates (45). Evidence points to the transformation of a single precursor cell that proliferates to form a clone since all cells in a tumor appear to share some particular characteristic(s) of the original precursor cell (45).

Cancer cells are defined by two properties. The first property is that they and their progeny reproduce without regard of normal constraints; the second property is that they invade and colonize areas that are needed by other cells (44). Differentiation is impaired although considerable differentiation may occasionally be observed in cancer allowing for the recognition of the tissue of origin (45).

Out of control proliferation give rise to a tumor, or neoplasm, which can either stay clustered or can invade surrounding tissue. The ability to invade other tissue depends on the ability to break loose, enter the bloodstream or lymphatic vessels, and form secondary tumors, or metasteses (44,45). There are several mechanisms that ensure that cell proliferation does not exceed the needs of a normal tissue. To name a few, there is promotion of cell differentiation by tumor suppressor genes, limitation of the total number of cell generation before senescence and apoptosis (46). Numerous normal developing tissues eliminate improperly developing cells by initiating apoptosis (46).

The p53 gene, which is a tumor suppressor gene, appears to play a regulatory role in apoptosis (46). Activated p53 functions as transcriptional transactivator, transrepressor and participates in numerous protein-protein interactions (46). The ability of p53 to transcriptionally activate *apaf*-1 has been in mitochondria-induced apoptosis (46). Furthermore, activated p53 regulates the expression of genes that are in control of the mitochondrial membrane permeability, hence, the release of cytochrome c (46). Two sites in the *apaf*-1 promoter relative to the transcriptional start site were found to correspond to a p53-responsive element and a radiolabeled *apaf*-1 wild-type oligonucleotide containing the p53-responsive element was shown to bind p53 (46). A mutation in this gene, resulting in loss of function, prevents the physiological crucial elimination of unneeded cells (46). The p53 gene has been found inactivated in several

neoplasms (47).

The release of cytochrome c prior to membrane depolarization and its role in the formation of the apoptosome has been of interest in cancer research. As has already been mentioned earlier, cytochrome c is part of the electron transport chain. It also depends on other electron carriers of this chain for the transfer of electrons so that it can carry one electron at a time to Complex IV. What role these other electron carriers play in apoptosis regarding cancer treatment was investigated for this thesis.

Electron Carriers

Several electron carriers participate in the mitochondrial electron transport chain (figure 3, page 21) (32,33,44).

Complex I contains the NADH-coenzyme Q reductase (32,33,44). Nicotinamide adenine dinucleotide (NAD^+) is a major electron acceptor in oxidative (catabolic) pathways (32,33). It is involved in hydride anion transfer either to NAD^+ or from NADH (the reduced form) and because the hydride anion contains two electrons, acts solely as a two-electron carrier (32). NADH binds to the enzyme on the matrix side of the inner mitochondrial membrane and transfers its electrons to tightly bound flavin mononucleotide (FMN) (32). FMN belongs to the flavin coenzymes, can participate in one- and two-electron transfers and is a stronger oxidizing agent than NAD^+ (32). $FMNH_2$ then transfers electrons to a series of Fe-S proteins, which results in the two-electron transfers to coenzyme Q (32,33,44). Coenzyme Q is also known as ubiquinone, CoQ or UQ (32). It owes it hydrophobicity to its isoprenoid tail, which allows it to freely diffuse in the inner mitochondrial membrane (32). This allows the shuttling of electrons between Complex I, II and III (32).

In complex II, succinate dehydrogenase contains two Fe-S proteins, flavoprotein 2 that has an FAD covalently bound to a histidine residue and three Fe-S centers. FAD is reduced to $FADH_2$.

Complex III, CoQ- cytochrome c reductase, has three different cytochromes and one Fe-S protein. The cytochromes are: *b* cytochrome (b_L and b_H) and *c* cytochrome. They are one-

electron carriers. It is only cytochrome c that transfers electrons from Complex III to Complex IV (32). Electrons traveling through Complex III are passed through cytochrome c_1 to cytochrome c (32,33). Cytochrome c is the only watersoluble one of the cyochromes and like ,UQ, a mobile electron carrier. It associates loosely with the inner mitochondrial membrane, in the IMS on the cytosolic side of the inner membrane, to aquire an electron from the Fe-S-cyt c_1 aggregate of Complex III (32,33). It migrates from Complex III along the membrane surface in the reduced state to Complex IV (32,33).

Complex IV, Cytochrome c Oxidase, consists of cytochrome c a and a_3 as well as two copper atoms. The reduction of molecular oxygen to water is accomplished here.

In summary the major electron carriers of the respiratory chain are: NADH, coenzyme Q, cytochrome c and FAD. Table 2 shows their standard reduction potentials.

Table I

Alphabetical list for amino acid abbreviations

One-letter Symbol	Three-letter Abbreviation	Amino Acid
A	Ala	Alanine
B	Asx	Asparagine or aspartic acid
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine
Z	Glx	Glutamine or glutamic acid

Table II

Standard reduction potentials of the four major electron carriers of the respiratory chain

Oxidant	Reductant	Transferred e^-	E'_o(V)
Cytochrome c (+3)	Cytochrome c (+2)	1	+ 0.254
CoQ (oxidized)	CoQ (reduced)	2	+ 0.060
FAD^+	$FADH_2$	2	- 0.219
NAD^+	$NADH + H^+$	2	- 0.320

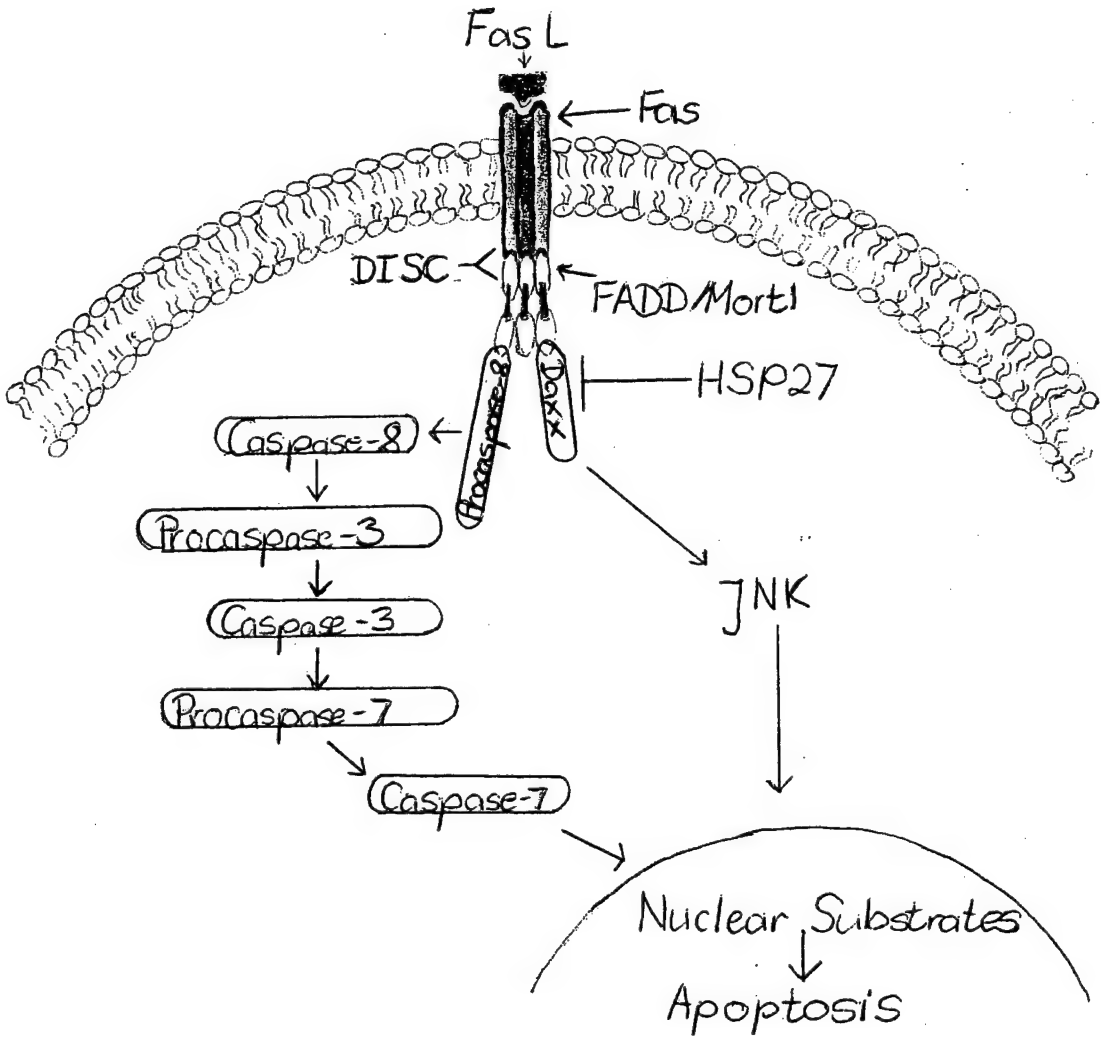

FIG. 1 Death receptor apoptosis. Binding of FasL (Fas/CD95 ligand) to Fas receptor induces trimerization of Fas. The death domain-containing (DD-containing) adaptor molecule, FADD/Mort1, contains a DD at its C-terminus, is recruited to the cytoplasmic region of Fas and binds to the DD of Fas forming death-inducing signaling complex (DISC). The N-terminus of FADD is needed for recruiting initiator procaspase-8 and/or procaspase-10. Procaspase-8 has two death effector domains (DEDs) at its N-terminal region where it binds FADD. Procaspase-8 autoactivates at the DISC, then activates procaspase-3, which is the procaspase that death receptor and mitochondrial-dependent apoptosis pathways converge on. Daxx can associate with the Fas receptor and activate the JNK apoptotic pathway.

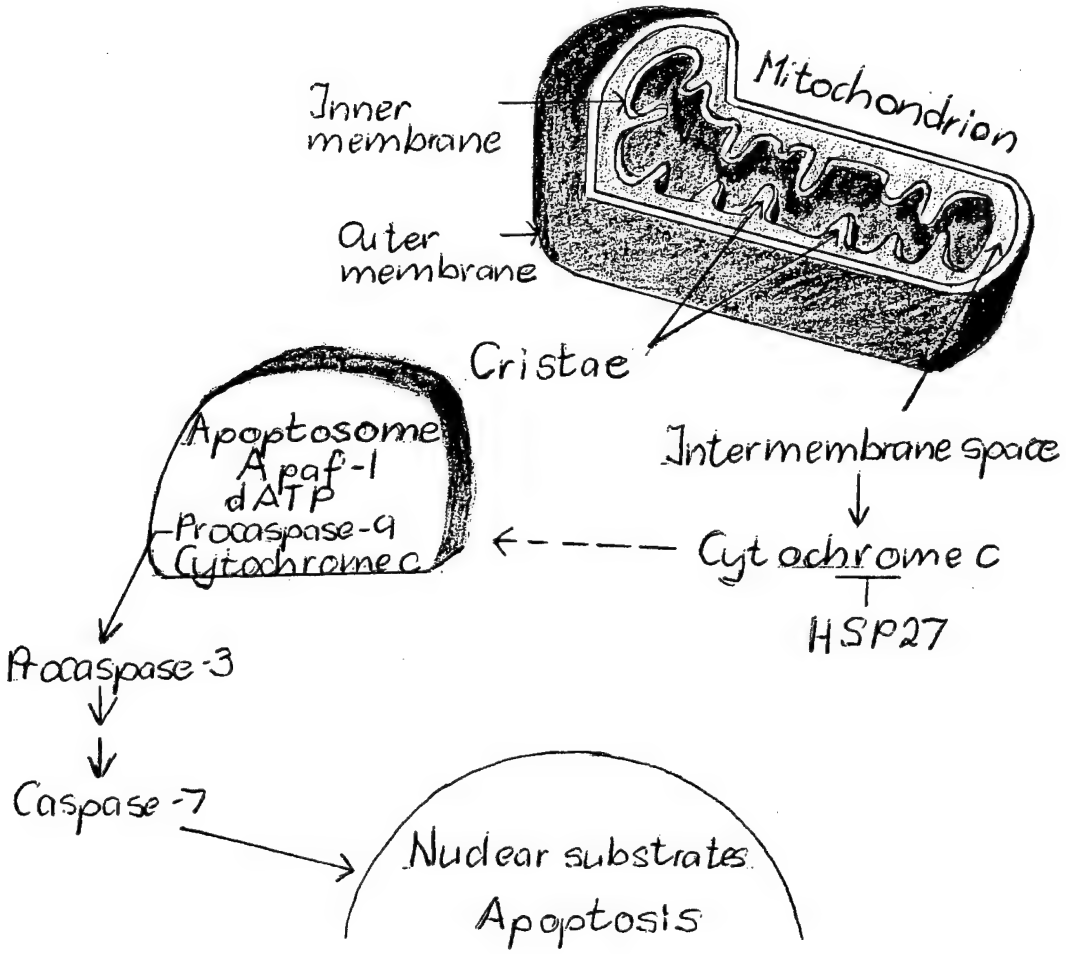

FIG. 2 Mitochondrion-dependent apoptosis. In response to apoptotic stimuli the mitochondrion releases cytochrome c, caspases, AIF (apoptosis-inducing factor), and SMAC (DIABLO) (second mitochondria-derived activator of caspases and murine homologue) into the cytosol to trigger caspase activation. In the cytosol cytochrome c is essential to the apoptosome, which consists of *apaf*-1, dATP and procaspase-9. Procaspase-9 autoactivates at the apoptosome, then cleaves procaspase-3, which cleaves procaspase-7, which cleaves specific target substrates leading to apoptosis. The binding of HSP27 to cytochrome c prevents the formation of the apoptosome; therefore inhibiting apoptosis.

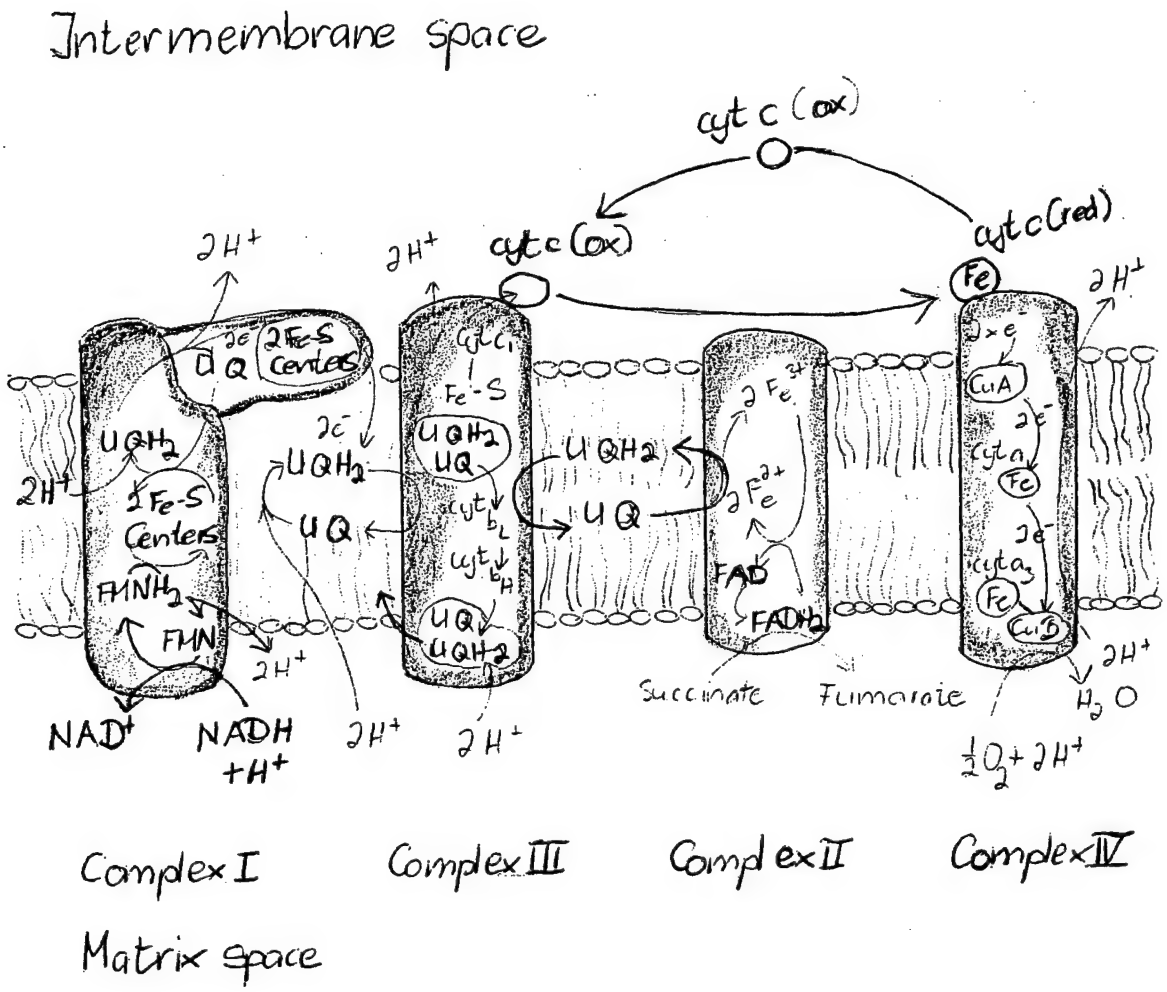

FIG. 3 The mitochondrial electron transport chain (respiratory chain). Four distinct protein complexes make up the mitochondrial respiratory chain: (I) NADH-coenzyme Q reductase, (II) succinate-coenzyme Q reductase, (III) coenzyme Q-cytochrome c reductase, and (IV) cytochrome c oxidase. NADH donates electrons to Complex I, serving as a link between glycolysis, the TCA cycle, fatty acid oxidation and the electron transport chain. Complex I and II produce a common product, reduced coenzyme Q (UQH_2), which becomes the substrate for Complex III. Complex III oxidizes UQH_2 and reduces cytochrome c, which is the substrate for Complex IV and this is where molecular oxygen is reduced to water.

CHAPTER 2

MATERIALS AND METHODS

Cell Lines

Four human breast cancer cell lines (DC4, DB46, MCF-7 & MDA-MD-435), one human colon cancer cell line (RKO) and one normal, human lung fibroblast cell line (HFL 1) were used. RKO and HFL1 were a kind gift from Dr. R. Gary (UNLV). DC4 & DB46 were derived from an estrogen-receptor negative human breast cancer cell line MDA-MD-231 (7). DB46, which has the constitutive expression vector pβ27, expresses elevated levels of heat shock protein 27. The vector was constructed by cloning a *Eco*RI cDNA fragment of HSP27 into the *Sal*I site of pHβApr-1-neo by a blunt end ligation after filling in the fragments (48). DC4, which is the control, contains only the vector pHβApr-1-neo; therefore, it expresses basal levels of heat shock protein 27 (7). MCF-7 has cytoplasmic estrogen receptors and MDA-MD-435 does not (9). All cell lines were grown in Minimal Essential Media (MEM), supplemented with 10% fetal bovine serum, 25 m*M* HEPES buffer (pH 7.4), 100IU penicillin/ml, 100 μg/ml streptomycin, 2 m*M* L-glutamine, and for DC4, DB46 cell lines, 400 μg/ml G418 sulfate. RKO was supplemented with 1.0 m*M* sodium pyruvate. All reagents used in cell culture were purchased from Gibco BRL, Grand Island, NY. All cell lines were maintained in humidified incubators at 37°C with 5% CO_2:95% air. Forty-eight hours before an experiment the cell lines, DC4 & DB46, were washed and resuspended in supplemented MEM lacking G418.

Electron Carriers and Reagents

The following electron carriers and reagents were purchased from Sigma: Cytochrome c, Q_{10}, DMSO, FAD, β-NAD, ATP. $MgCl_2 \cdot 2H_2O$ was obtained from Fisher Scientific.

Cytochrome c was suspended in 5 ml of PBS (phosphate buffered saline, pH 7.2, (1X), no Mg^{+2}, no Ca^{+2}) from which serial dilutions were made to yield experimental concentrations (PBS was purchased from Gibco BRL). FAD, β-NAD, ATP and $MgCl_2$ were suspended in 10 ml of PBS from which dilutions were made. $MgCl_2$ was used as a control in the ATP experiment. It was used at $1x10^{-7}$M, which was the highest experimental concentration of ATP utilized. Q_{10} was suspended in heated DMSO. A beaker was filled with enough H_2O to submerge half a 15 ml centrifuge tube containing DMSO and Q_{10}. H_2O was heated for one minute in a microwave on high power. After removing the beaker from the microwave the 15 ml centrifuge tube was submerged half-way for 30 seconds at a time (3x). After each submersion the 15ml microfuge tube was vortexed. After the third vortex Q_{10} was dissolved. DMSO was used as a control in the following molarities: 276mM, 138mM, 27.6 mM and 2.76 mM. All reagents and electron carriers were made fresh and filter sterilized prior to dilutions.

Heat Shock

Twenty-four hours before the experiment, a water bath was filled with deionized H_2O and heated to 45°C ±0.01°C. A DC4 flask (T-25) was sprayed with 70% ethanol, taken under the hood and its vented cap replaced with a solid cap. The entire flask was wrapped with parafilm so that no water could enter the flask once it was submerged in the water bath. The flask was then submerged in the heated water for 10 minutes after which it was removed. Parafilm was removed, Kim wipes were used to collect any water that might have gotten into the cap, flask was sprayed with 70% ethanol and taken under the hood. Then the cap was exchanged and secured. The flask was then transferred back to the incubator.

Clonogenic Survival

The cell lines were exposed to the above mentioned electron carriers and reagents in various concentrations. Each exposure was for one hour at 37°C after which the cells were washed once in 5 ml of PBS, trypsinized, resuspended in supplemented MEM and counted using a Coulter

Counter model ZF. Cells were plated in 60 mm tissue culture dishes. Each experiment was set up in triplicates. Cells were incubated at 37°C in a humidified incubator with 5% CO_2:95% air for eleven days. After incubation, cells were removed from incubator, stained with crystal violet (5% Crystal violet:95% ethanol), allowed to dry and counted. Colonies consisting of > 50 cells were counted (cells survived). Results are graphed as percent survival.

Western Blotting

Samples (protein or lysed, which cell lines) were run on SDS polyacrylamide gel electrophoresis. The (results) were transferred onto a nitrocellulose membrane. Western blotting was performed using (which antibodies) to HSP27. (This section will be finished a. s. a.p.)

Statistics

Will be done in the very near future.

CHAPTER 3

RESULTS

Cytochrome c Induces Cell Death

The effects of exogenous addition of various cytochrome c concentrations were investigated. Cell lines DC4, DB46, MCF-7, MDA-MD-435 were exposed to the following concentrations of cytochrome c: 0, $5x10^{-7}$M, $5x10^{-6}$M and $5x10^{-5}$M (figures 4, 5). DC4 was heat shocked as well for this experiment to see if more protection from cytochrome c toxicity could be observed. RKO and HFL1 were exposed to these cytochrome c concentrations: 0, $5.6x10^{-8}$M, $5.6x10^{-7}$M and $5.6x10^{-6}$M (figure 6).

DC4, HS DC4 and DB46 cells showed a does response. Cell survival was most affected in response to the highest concentration of cytochrome c $5x10^{-5}$M. HS DC4survival rate was similar to DB46.

MCF-7 and MDA-MD-435 also responded in a dose dependent manner with the most cell kill observed at $5x10^{-5}$M. MCF-7 had the highest survival rate of all the breast cancer cell lines. This may be explained by constitutive expression of HSP27 in the cell line.

RKO was not sensitive to any of the cytochrome c concentrations and HFL1 only was sensitive at the highest cytochrome c concentration with about a 45 % cell survival. In these cases it is not clear what mechanism was induced to protect these two cell lines against apoptosis. It is clear; however, that they were much more resistant to cytochrome c toxicity.

In summary, the survival rates at the highest cytochrome c concentrations are as follows: RKO - 100 %, HFL1 - 55.3 %, MCF-7 - 64.5 %, HS DC4 – 44 %, DB46 - 41 %, MDA-MD-435 - 36 % and DC4 - 18 %.

FAD Can Induce Cell Death

This lab has shown that cytochrome c is cytotoxic in breast cancer cell lines. Given that cytochrome c is an elelctron carrier involved in the mitochondrial electron transport chain, the decision was made to look at the other electron carriers that play a role in this electron transport chain. Fad was therefore used in this experiment. The same cell lines as in the above experiment were used (figures 7, 8, 9). DC4 was not heat shocked for this experiment.

FAD was applied in the following concentrations: $1x10^{-9}M$, $1x10^{-8}M$ and $1x10^{-7}M$. These concentrations are much smaller than the cytochrome c concentrations; yet, FAD was found to be more toxic than cytochrome c .

The survival rates at the highest FAD concentration are as follows: RKO - 74.9 %, MCF-7 - 63.8 %, HFL1- 61.08 %, DB46 - 57.2 %, MDA-MD-435 - 41.0 % and DC4 - 30.2 %.

The mechanism of cell death following FAD exposure is not known. How or if FAD enters the cell is not known.

ATP Can Affect Cell Survival

FAD cell toxicity was not an anticipated outcome of the above experiment. ATP consists of AMP and two attached phosphates. FAD and ATP both have an AMP moiety. This observation was the catalyst to use ATP in this experiment using the same experimental concentrations as in the FAD experiment. $MgCl_2$ was used as a control to make certain it does not induce cell death. The concentration for $MgCl_2$ was $1x10^{-7}M$ and results showed that it did not have any effect on the cells. DC4 and DB46 were the only cell lines used in this experiment given the trend of similar cell survivals among the breast cancer cell lines and keeping in mind that the research is pertaining to breast cancer research (figure 10). ATP was not able to elicit a dose response in the cell lines. Why this happened is not understood.

NAD^+ Can Induce Cell Death

Since FAD and cytochrome c are able to induce cell death and are both electron carriers, we were interested in the toxicity of another electron carrier. NAD^+, like FAD has an AMP moiety attached to its structure. The concentrations were the same as in the FAD and ATP and the cell lines used were DC4, DB46, RKO and HFL1.

NAD^+ did not produce a dose response but also seems to be toxic in DC4 and DB46. At the highest concentration cell survival is 38.1 % and 39.9 % respectively. It seems to be more toxic in the DB46 cell line than the DC4 cell line compared to FAD with 30.2 % and 57.2 % cell survival in DC4 and DB46 respectively.

RKO cell proliferation seemed to be stimulated after NAD^+ exposure. HFL1 showed a dose response as concentrations were increased with a 71.0 % cell survival at the highest concentration (figures 11, 12).

How cell death is induced with this electron carrier is not known. The same concentrations yielded different responses in the cell lines; therefore, the only conclusion that can be drawn at this time is that somehow NAD^+ is cytotoxic in some cell lines.

Q_{10} May Induce Cell Death

The last electron carrier of the electron transport chain left to investigate was Q_{10}. DMSO was used as a control as described in chapter 2 (Materials and Methods) and was found not to affect cell survival (figure 13). Concentrations were kept the same as in the FAD and NAD^+ experiments. DC4 and DB46 displayed cell death only at the highest concentration with cell survival of 53.4 % and 74.5 % respectively. RKO and HFL1 were both resistant to all of the concentrations applied (figure 13).

The conclusion drawn from this experiment is that Q_{10} is the least effective of all three electron carriers in cell death induction.

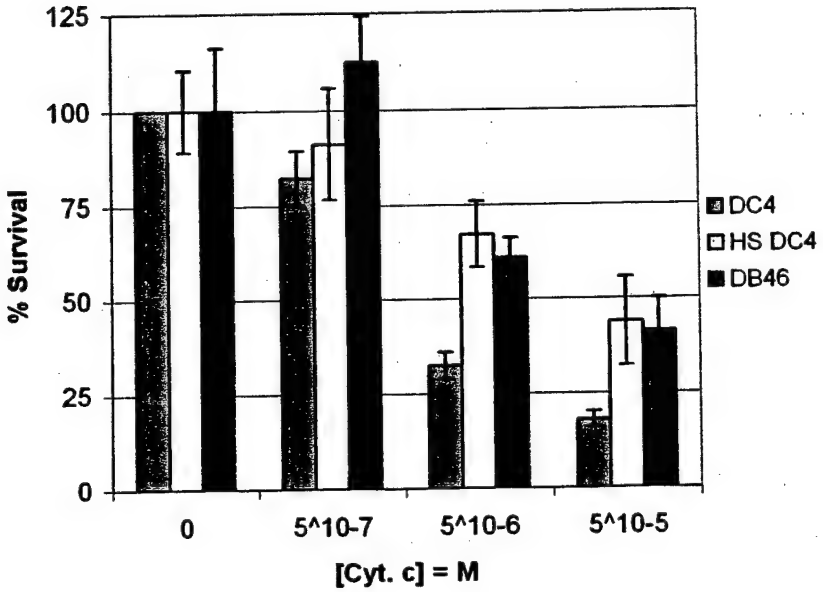

FIG. 4. Cytochrome c toxicity observed in DC4, HS DC4 and DB46 cell lines. Increasing concentrations of cytochrome c elicit a dose response (lowest percent survival at highest concentration) in DC4, HS DC4 and DB46.

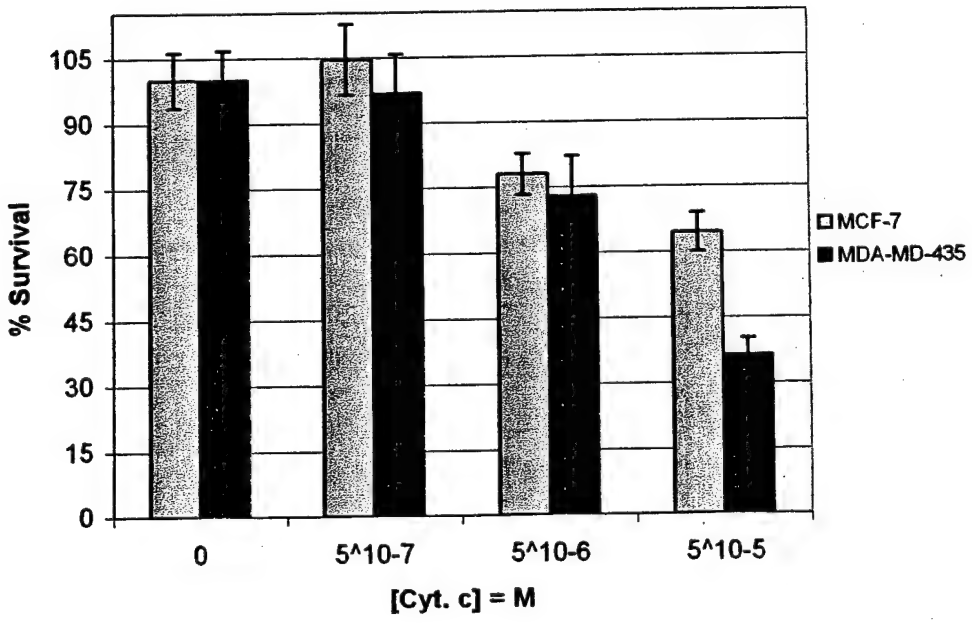

FIG. 5. Cytochrome c toxicity observed in MCF-7 and MDA-MD-435. A dose response is observed in both cell lines with the most cell kill at the highest concentration. MCF-7 was more resistant to cytochrome c exposure than MDA-MD-435.

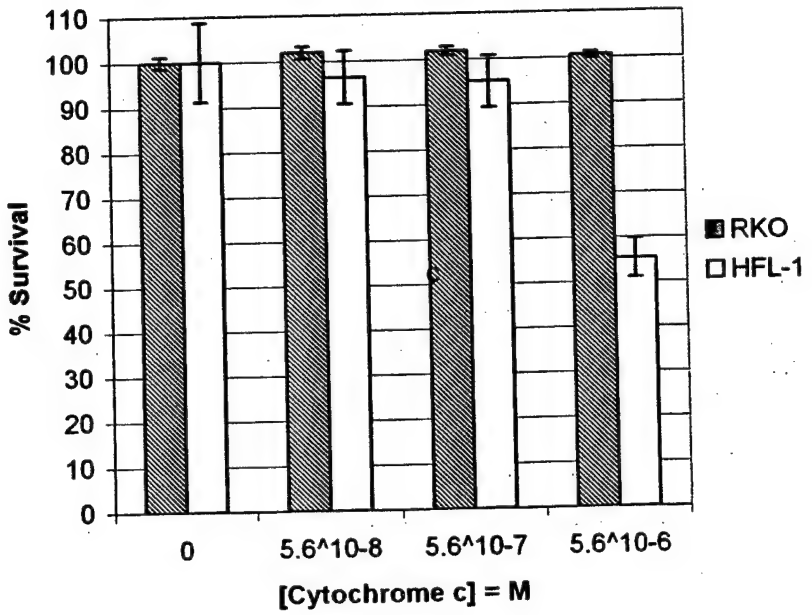

FIG. 6. Cytochrome c toxicity observed in RKO and HFL1. RKO is resistant to all concentrations of cytochrome c. HFL1 is sensitive to cytochrome c only at the highest concentration.

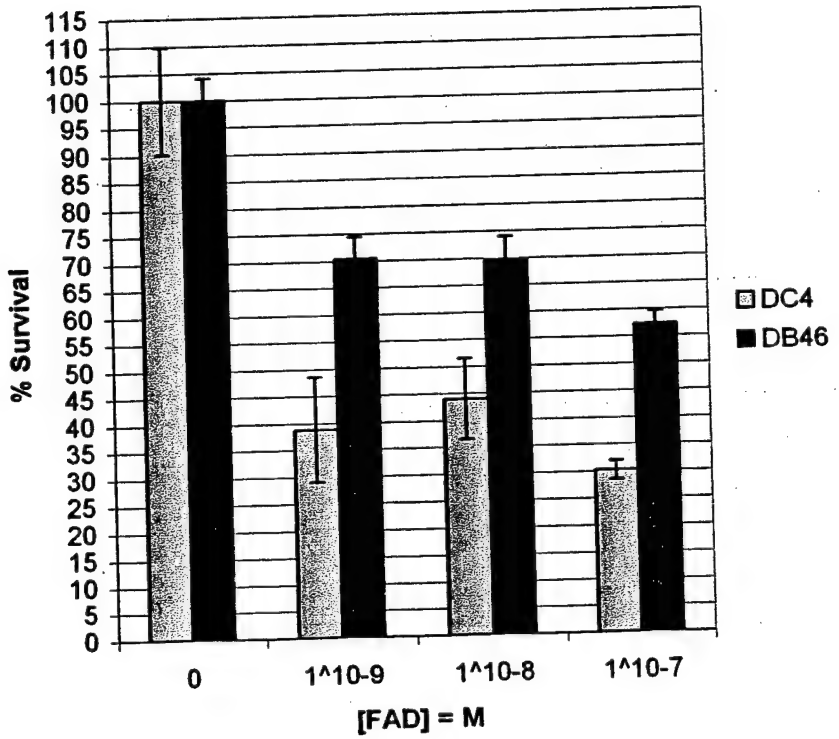

FIG. 7. FAD toxicity observed in DC4 and DB46. All concentrations have an effect on both cell lines. DC4 is more sensitive than DB46 to incubation with FAD. The highest concentration of FAD yields 70% cell kill in DC4 and 48% cell kill in DB46.

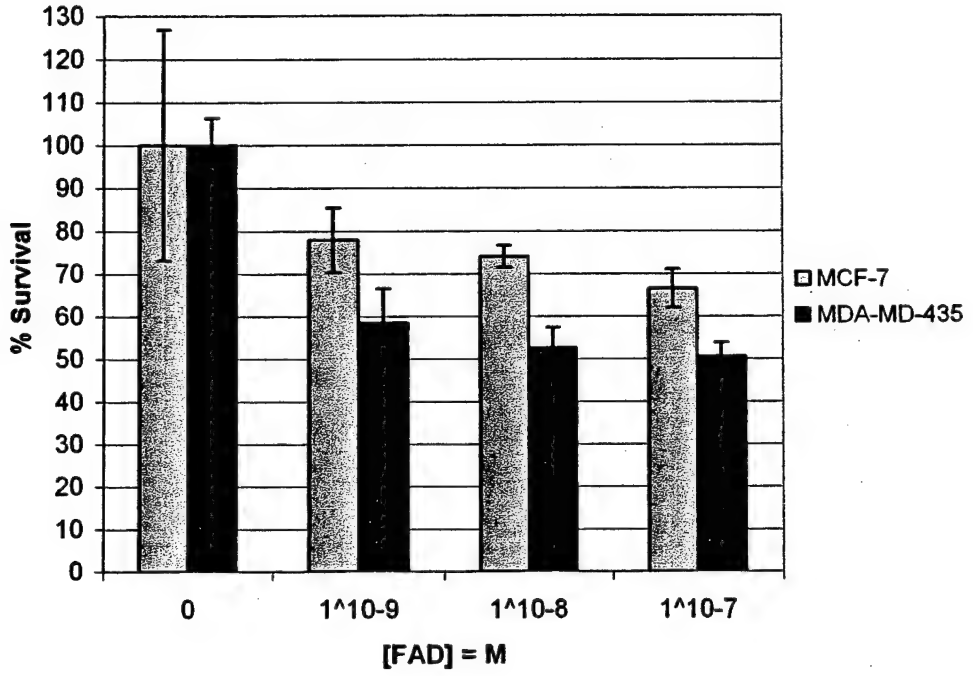

FIG. 8. FAD toxicity observed in MCF-7 and MDA-MD-435. A dose response to incubation with FAD is observed in both cell lines with the lowest survival occurring at the highest concentration of FAD.

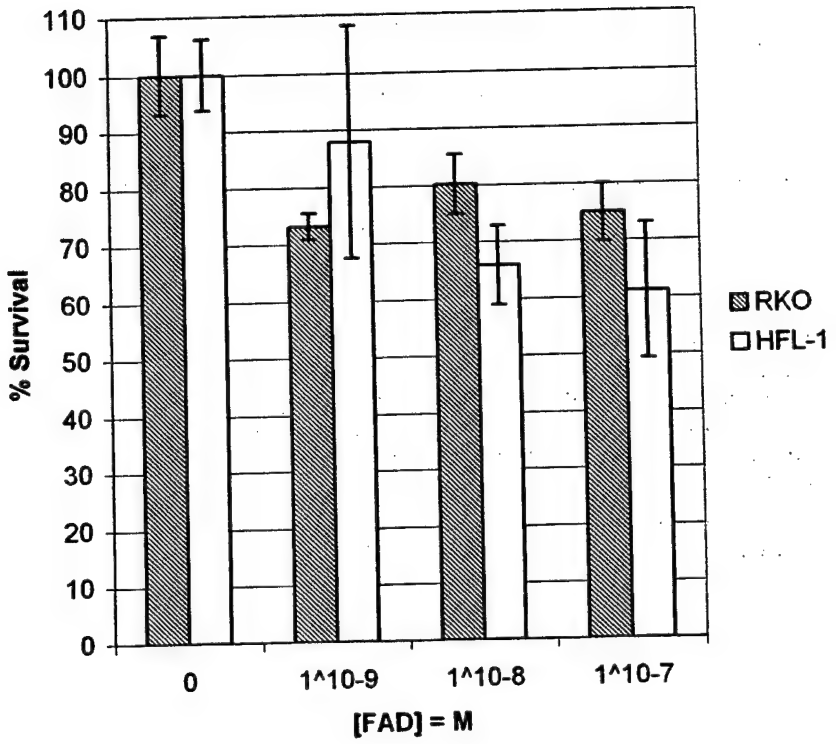

FIG. 9. FAD toxicity observed in RKO and HFL1. FAD has effects on both cell lines. RKO responds to incubation with FAD but holds its survival rate steady above 70%. Incubation of HFL1 with FAD shows a dose response. The lowest cell survival is observed at the highest concentration of FAD.

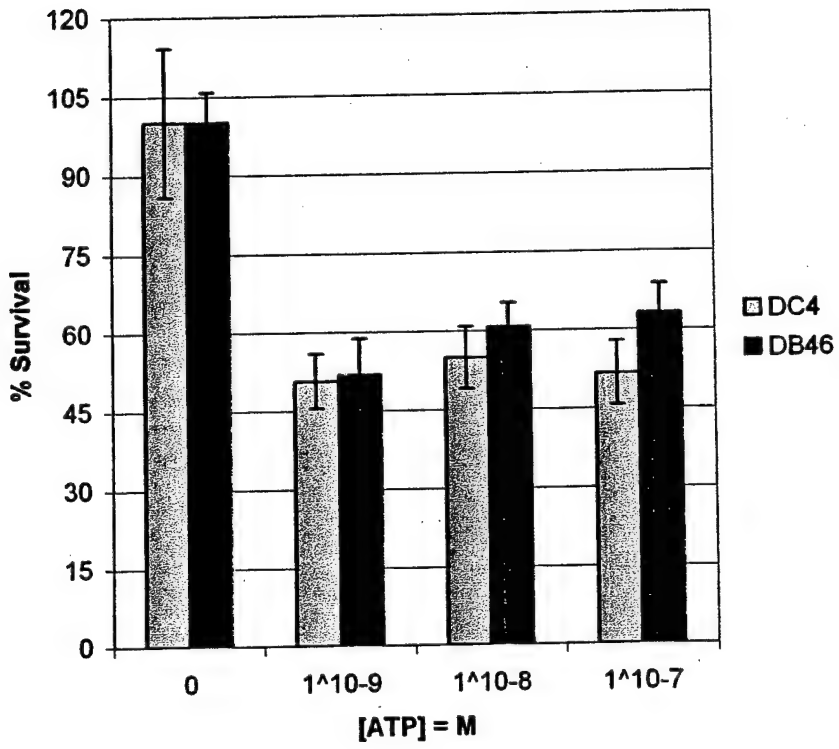

FIG. 10. ATP toxicity observed in DC4 and DB46. DB46 and DC4 respond to incubation with ATP. A dose response is not observed in either cell line. DB46 is somewhat more resistant than DC4 to incubation with ATP but both cell lines have a similar pattern.

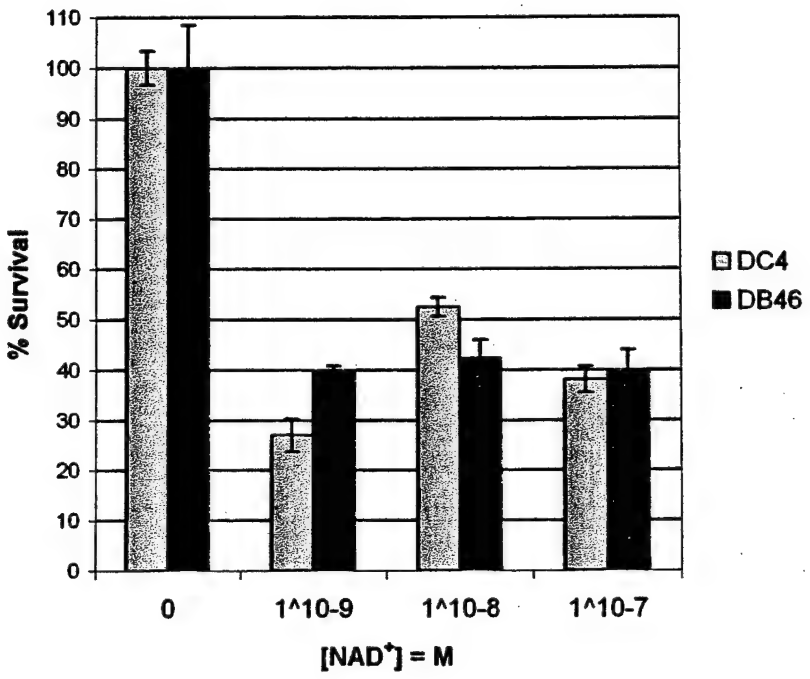

FIG. 11. NAD^+ toxicity observed in DC4 and DB46. Incubation of DC4 and DB46 with NAD^+ results in cell kill. A dose response is not observed in either cell line.

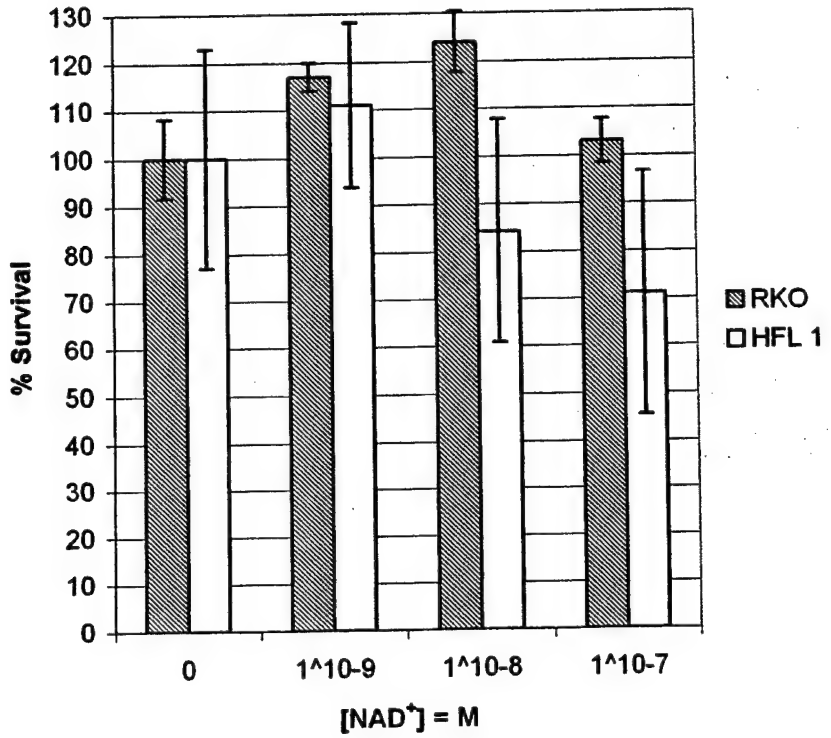

FIG. 12. NAD^+ toxicity observed in RKO and HFL1. NAD^+ appears to stimulate proliferation in RKO. A dose response to NAD^+ is seen in HFL1. The highest concentration results in the lowest cell survival.

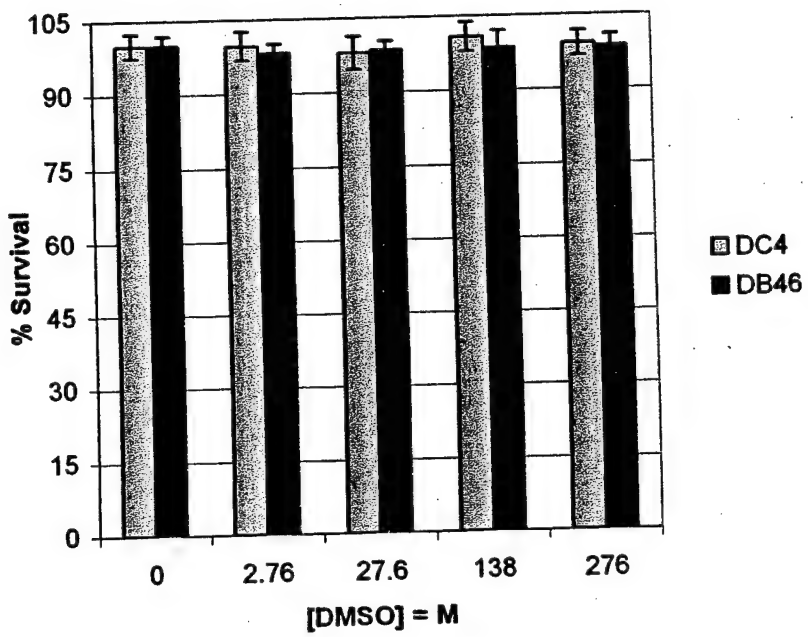

FIG. 13. Incubation of DC4 and DB46 with DMSO. DMSO did not have an effect on either cell line.

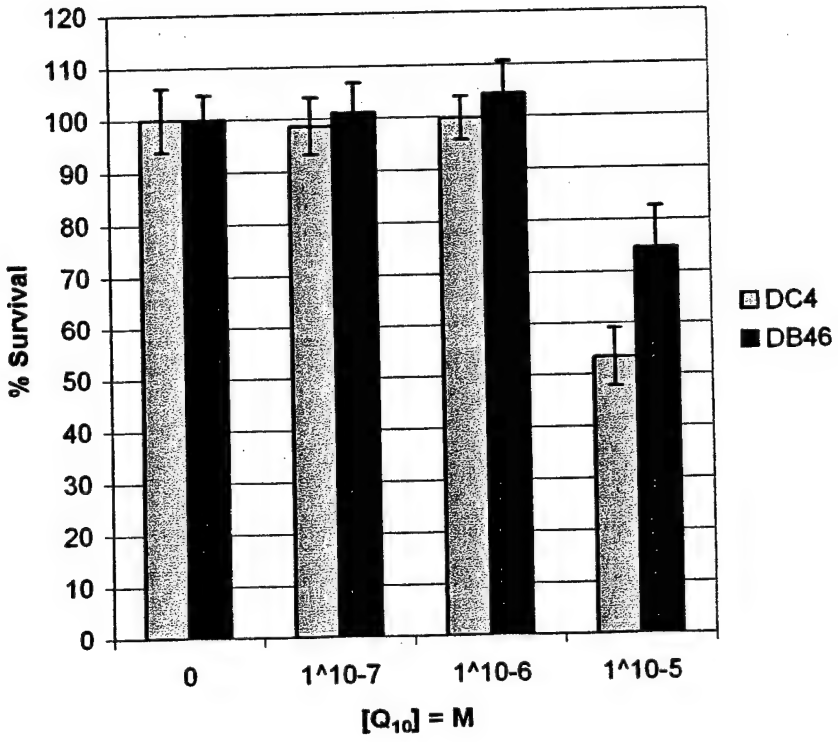

FIG. 14. Q_{10} Toxicity observed in DC4 and DB46. Q_{10} showed an effect only at the highest concentration in both cell lines. A dose response is not observed.

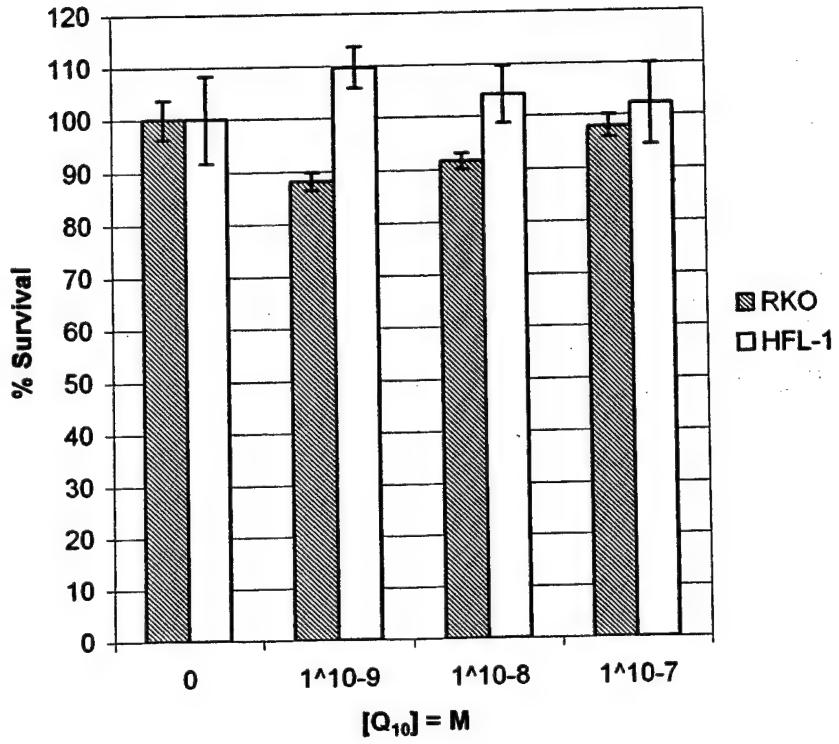

FIG. 15 Q_{10} toxicity observed in RKO and HFL1. Q_{10} has no effect on HFL1. RKO is most sensitive at the lowest concentration only. A dose response is not observed in either cell line.

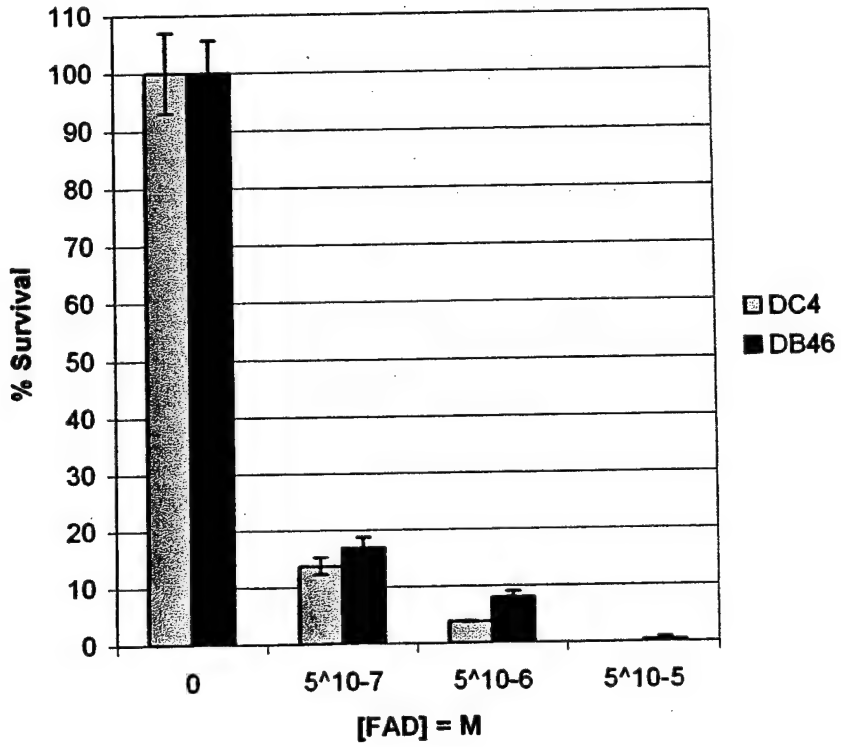

FIG. 16 FAD toxicity observed in DC4 and DB46. FAD applied in the same concentrations as cytochrome c overwhelms both cell lines. DC4 has no cell survival at the highest concentration of FAD.

CHAPTER 4

DISCUSSION

Previous research has given indirect evidence that HSP27 binds to cytochrome c (5). This binding prevents the formation of the apoptosome and thus inhibits apoptosis. The goal of this thesis is to investigate the effects of exogenous addition of cytochrome c to cells. Can cytochrome c overcome the inhibition of HSP27 and therefore induce apoptosis? Given that cytochrome c is an electron carrier involved in the mitochondrial electron transport chain, do other electron carriers, which are also involved in this electron transport chain, have cytotoxic effects on cells as well? To answer these questions the following experiments were set up and the results of these experiments are discussed in the following paragraphs.

Cytochrome c toxicity was investigated using the cell lines DC4, DB46, MCF-7, MDA-MD-435, RKO and HFL1. The first experiment was set up with DC4, HS DC4 (heat shocked DC4) and DB46 (figure 4, page 29). DC4 was heat shocked, which results in the induction of many proteins that are involved in cell rescue, including HSP27. Cytochrome c was applied in three different concentrations ($5x10^{-7}$M, $5x10^{-6}$M, $5x10^{-5}$M) for one hour each, which elicited a dose response. As mentioned in chapter 2 (Materials and Methods) DB46 has elevated levels of HSP27. In this experiment HS DC4 responded in a cell survival that was similar to DB46. The cell survivals were 41% and 44% respectively. For HS DC4, this could indicate that among other heat shock induced proteins, elevated levels of HSP27 were present as well. As expected, DC4 was not able to tolerate cytochrome c toxicity well with 18% survival. This can be attributed to the basal levels of HSP27, which were not enough to protect the cells form the toxic effects of cytochrome c.

The next cell lines exposed to exogenous cytochrome c were MCF-7 and MDA-MD-435. These two cell lines also responded in a dose dependent manner (figure 5, page 30). MCF-7

was more resistant to cytochrome c exposure than MDA-MD-435. At the highest concentration, $5x10^{-5}$ M, MCF-7 had 65% cell survival, which is about 30% more survival than in MDA-MD-435. It can be speculated that one of the factors of more cell survival is due to constitutive expression of HSP27, which is not the case in MDA-MD-435.

RKO was resistant to all cytochrome c concentrations ($5x10^{-8}$M, $5x10^{-7}$M and $5x10^{-6}$M) with 100% survival at all concentrations and HFL1 was sensitive to cytochrome c only at the highest concentration $5x10^{-6}$M with 55% cell survival (figure 6, page 31). What mechanism(s) RKO cells have to protect against cytochrome c toxicity or what its levels of HSP27 are is not known. It is not clear why only the highest dose of cytochrome c had an effect in the HFL1 cell line.

In summary, these experiments showed that in breast cancer cell lines, increasing concentrations of cytochrome c could overcome the inhibitory effects of HSP27 and induce apoptosis. It was also demonstrated that not all cancer cell lines may be sensitive to cytochrome c and that some normal cell lines can be affected by exogenous cytochrome c addition.

FAD is involved in the mitchondrial electron transport chain and therefore was investigated for toxicity in DC4 & DB46 (figure 7, page 32) and in the other four cell lines. The concentrations, $1x10^{-9}$M, $1x10^{-8}$M, $1x10^{-7}$M, were used. DC4 and DB46 were responding in a dose dependent manner. At the highest concentration DB46 had a 57% cell survival giving it about 27% more cell survival than DC4. It was shown in the cytochrome c experiment that elevated levels of HSP27 gave protection against the toxic effects of cytochrome c. As mentioned previously, increasing concentrations of cytochrome c were able overcome this inhibition; thus, a correlation may possibly be made. Increasing concentrations of FAD were able to overcome the inhibitory effects of HSP27 and therefore induce cell death just as cytochrome c did. DC4 had no protection against cell death, which can be explained by the basal levels of HSP27 in this cell line.

MCF-7 and MDA-MD-435 also showed a dose response to this electron carrier (figure 8, page 33). The concentrations of FAD were the same ($1x10^{-9}$M, $1x10^{-8}$M, $1x10^{-7}$M) and at the highest concentration, MCF-7 had 64% cell survival, which was about 25% more cell survival than what MDA-MD-435 displayed. It can be speculated that HSP27 inhibited cell death.

FAD did have an effect on RKO and HFL1 (figure 9, page 34). Cell survival was 75% and 61% respectively. Somehow FAD can trigger pathways that induce cell death in these cell lines. The mechanisms remain to be investigated.

To summarize the results of the FAD experiments, it is remarkable that FAD is toxic at much lower concentrations than cytochrome c. How this electron carrier enters the cell is not understood. What other factors such as the expression of other heat shock proteins involved in cell rescue that may contribute to cell survival after FAD treatment is also not known. No studies have shown that HSP27 binds FAD and no studies have been done on FAD toxicity.

The unexpected results of the FAD toxicity were the catalyst behind the investigation of ATP toxicity. FAD and ATP both have an attached AMP moiety. This lead to the reasoning, that perhaps, FAD was utilizing an extracellular ATP receptor thereby gaining entry into the cell. DC4 and DB46 cell lines were used and the same concentrations as in the FAD experiments were applied (figure 10, page 35). These two cell lines did not show a dose response. The highest concentration appeared to stimulate cell proliferation in DC4 (80%) and DB46 (89%). DC4 showed 65% cell survival at $1x10^{-9}$M and 70% at $1x10^{-8}$M whereas DB46 showed cell survival of 60% at $1x10^{-9}$M and 62% survival at $1x10^{-8}$M. What caused such a sudden drop in cell survival followed with cell proliferation at the highest concentration is not understood. It did make it clear though that ATP at these concentrations did not induce cell death as effectively as FAD. How ATP induced cell death at the lower concentrations remains to be investigated.

The next electron carrier involved in the mitochondrial electron transport chain with an attached AMP moiety is NAD^+. Concentrations were held the same as in the FAD and ATP experiments. The exogenous addition of NAD^+ to cell lines DC4 and DB46 resulted in cell death (figure 11, page 36). It is peculiar that the lowest concentration, $1x10^{-9}$M, yielded the lowest cell survival of 27%. This was a similar occurrence in the FAD experiment. The next concentration, $1x10^{-8}$M, almost doubled the cell survival to 52% and the highest concentration, $1x10^{-7}$M, resulted in survival reduction to 38%. DB46 had 40% cell survival at $1x10^{-9}$M, 42% cell survival at and 40% cell survival at $1x10^{-7}$M. Increasing NAD^+ concentrations could not influence the survival

rates in DB46. NAD^+ is more effective in cell kill at lower concentrations than FAD and therefore the RKO and HFL1 cell lines were tested next.

RKO and HFL1 were given the same concentrations of NAD^+ (figure 12, page 37). RKO was not sensitive to any of the NAD^+ concentrations but instead showed an increase of cell proliferation to 117% at $1x10^{-9}M$ and 124% at $1x10^{-8}M$ and down to 103% at $1x10^{-7}M$. HFL1 did show a dose response in decreasing cell survival from 111% to 84% to 71% as concentrations increased.

To summarize the results of the NAD^+ experiments, it was found that this electron carrier may be more effective at cell kill in breast cancer cells at lower concentrations than FAD. A dose response was not observed. The concentrations resulted in a sudden drop of cell survival in these cell lines. Not all cancer cell lines may respond in the same fashion. The RKO cell line was resistant to all applied concentrations. On the other hand, a normal cell line can show a dose response as was observed in HFL1.

Seeing that the three electron carriers that are involved in the respiratory chain have effects on cell survival, the fourth electron carrier that is part of this chain begged to be investigated as well.

Q_{10} had to be dissolved in DMSO (chapter 2, page 23). DMSO was therefore set up as a control to show that it was not able to induce cell death (figure 13, page 38). The results of the experiment were as expected. DMSO did not induce cell death. With this conformation, Q_{10} was added exogenously in the following increasing concentrations, $1x10^{-7}M$, $1x10^{-6}M$, $1x10^{-5}M$, to the cell lines DC4 and DB46. Only the highest concentration showed to have an effect on DC4 and DB46 with cell survival being 53% and 74% respectively.

The RKO and HFL1 cell lines were also checked for Q_{10} toxicity (figure 15, page 40). The concentrations $1x10^{-9}M$, $1x10^{-8}M$, $1x10^{-7}M$ were not able to induce any significant cell death in RKO. The lowest concentration showed 88% cell survival, $1x10^{-8}M$ showed 92% cell survival and $1x10^{-7}M$ showed 98% cell survival. These results indicate that RKO was resistant to the exogenous addition of Q_{10}. HFL1 showed resistance at any of the concentrations as well staying

at 100% survival.

The above results of Q_{10} can be summarized in one sentence. It appears that Q_{10} is the least effective of the four electron carriers of the mitochondrial electron transport chain to induce cell death in breast cancer cells as well as other cell lines.

Of all tested electron carriers in these experiments, cytochorme c and FAD were the only ones that seemed to elicit a dose response when applied exogenously to cells. FAD, as mentioned earlier, could do so at a much lesser concentration. One of the last questions left to answer then was what effect FAD had at the same concentration as cytochrome c. DC4 and DB46 were tested with the exogenous addition of FAD at $5x10^{-7}$M, $5x10^{-6}$M and $5x10^{-5}$M (figure 15, page 41). FAD turned out to be very toxic on these cell lines. DC4 had no cell survival at the highest concentration and DB46 showed only a 0.33% cell survival. These results show that FAD is more efficient than cytochrome c in inducing cell death.

To conclude the results of FAD, the experiments did show that FAD can elicit a dose response at a lower concentration than cytochrome c. What these experiments could not show was if FAD induces apoptosis or necrosis and how or if it enters the cell. Is there an extracellular receptor that FAD can bind to and be transported into the cell? Is there an extracellular receptor that FAD can donate electrons to that are then taken into the cell and this is what causes havoc resulting in cell death? These questions remain to be answered.

To conclude the cytochrome c experiments, it was shown that cytochrome c can induce apoptosis in breast cancer cell lines that constitutively express HSP27 or have HSP27 gene induction by heat in a dose dependent manner (figures 4, 5, pages 29,30). These results showed the hypothesis to be true. What these experiments did not show was whether HSP27 binds cytochrome c at the outer mitochondrial membrane as it leaves the mitochondria or if the binding takes place in the cellular milieu.

Future Work

There are several methods that could lead to the clarification regarding the effects of exogenously

added FAD. Co-immunoprecipitation could be investigated in the HSP27/FAD binding. This would answer the question whether or not HSP27 binds FAD therefore inhibiting cell death. There are several ways to identify apoptotic events in a cell. A flow cytometer can do a light scatter analysis. This application takes advantage of the refractive index. The refractive index of the internal cytoplasm of a dying cell becomes more similar to that of the extracellular medium. The addition of propidium iodide can identify those cells that have become permeable. A cell membrane analysis can be performed with a flow cytometer. Under normal circumstances phosphatidylserine (PS) residues are located in the inner membrane of the cytoplasmic membrane. These residues are translocated in the membrane and are externalized during apoptosis. Annexin –V is a specific PS-binding protein that can be conjugated to fluorochromes. A flourochrome can be combined with propidium iodide to identify dead cells. Enzyme analysis would show if FAD induces apoptosis. The investigation of caspase-8 and caspase-3 activity with assay kits can help determine which pathway of apoptosis becomes induced with FAD. Using a flow cytometer the active form of the enzyme could be detected by using a specific antibody. Another way would be to use a fluorochrome labeled peptide that binds to the active site of the caspase. Finally, using a non-fluorescent substrate for the caspase, which produces a fluorescent product if the enzyme is active, could help determine the initiated apoptotic pathway. Another method of apoptosis detection would be DNA analysis. Nucleases are activated during apoptosis that degrade DNA. One way to detect these DNA fragments would be to use strand break labeling (TUNEL). TUNEL stands for Terminal deoxynucleotidyl transferase mediated dUTP Nick End Labelling. An enzyme is used to add dUTPs to the broken ends of DNA, which can then be detected by antibodies with flourochrome labels. Staining with propidium iodide makes it possible to tell during which phase of the cell cycle the cells are exhibiting strand breaks.

Co-immunoprecipitation could be investigated in the HSP27/cytochrome c binding. Flourescent tagging of HSP27should help determine the location of the binding with cytochrome c.

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Appendix D

Molecular Chaperone Activities of HSP27

Appendix D Molecular Chaperone Activities of HSP27

Materials and Methods

Materials – All heat shock proteins were from a single commercial source (Stressgen, Vancouver B.C.). Alpha-glucosidase, aldolase, IgG, Trizma base, acetyl CoA, Hepes, MgCl2, ATP and p-nitrophenyl alpha-D-glucopyranoside (PNPG) were purchased from Sigma Chemical Company (St. Louis, MO). Citrate synthase, oxaloacetic acid and 5,5'-Dithio-bis [2-nitrobenzoesauve] (DTNB) were purchased from Boehringer Mannheim.

Alphaglucosidase assay – A 12.9 microM solution of alpha-glucosidase was prepared fresh in Hepes buffer (40 mM Hepes, pH [NaOH]) and stored on ice until used. Stock solutions were prepared by dilution of hsp27, hsp70, GroEL/ES with the Hepes buffer and stored on ice until ten minutes prior to the first assay. A 1.5 ml microfuge tube containing 170 microL of the alpha-glucosidase solution was removed from the ice and allowed to equilibrate at room temperature. Assays were performed by removing 30 microL aliquots and adding them to 3.0 ml of substrate solution and monitoring the absorbance at 440 nm. The substrate solution contained 2 mM PNPG in the Hepes buffer. Following the initial assay, samples were heat shocked at 45.0 C +/- 0.05 C for three minutes in a water bath. After the heating a 30 microL aliquot was immediately removed and assayed for alpha-glucosidase activity. The samples were maintained at room temperature and additional aliquots were assayed at the times indicated following the heat shock. Additions of proteins were made prior to or following the heat shock as indicated. Where indicated, 10 mM MgCl2 and 1 mM ATP (both prepared fresh) were added to the alpha-glucosidase solution prior to heating.

Citrate Synthase assay – A 1.44 microM solution of citrate synthase in Tris buffer (200 mM Tris, pH 8.1 [HCl]) was prepared fresh and placed on ice until used. Solutions of IgG and hsp27 were also prepared in Tris buffer and stored on ice until used. Ten minutes prior to the first assay, a 1.5 ml microcentrifuge tube containing 360 microL of citrate synthase solution was removed from the ice and allowed to equilibrate at room temperature. Assays were preformed by removing 30 microL aliquots and adding them to 3.0 mL of substrate solution and monitoring the absorbance at X nm. The substrate solution contained 200 mM Tris buffer, 2.3 X 10-4 oxaloacetic acid, 4.7 X 10-5 acetyl CoA, and 0.1 mM DTNB. Following the initial assay, samples were heat shocked at 45.0 C +/- 0.05 C for 25 minutes in a water bath. After the heating a 30 microL aliquot was immediately removed and assayed for citrate synthase activity. The samples were maintained at room temperature and additional aliquots were assayed at the times indicated following the heat shock. Additions of proteins or OAA were made prior to or following the heat shock as indicated.

Results

Figure 1 demonstrates that alpha-glucosidease has a thermo-labile enzymatic activity such that after five minutes of heating at 45° C reduces activity from 100% to approimately 30%. This decreased activity does not spontaneously recover but remains at the same level for at least four hours following the heat shock (data not shown).

Addition of the enzyme aldolase prior to heating did not help protect the enzymatic activity of alpha-glucosidase. However, addition of hsp27 prior to heating results in a protection of approximately 20%. This protection is dose dependent such that the greater the amount of hsp27 added prior to heat shock, the greater the protection.
A second enzyme, citrate synthase, was used to confirm our results with alpha-glucosidase. Figure 5 demonstrates that if 1.4 μM hsp27 is added following a heat shock it is unable to help citrate synthase regain enzymatic activity. But if hsp27 is added prior to a heat shock it is able to protect the enzymatic function (by about 25%) of citrate synthase IgG was used as a control protein and was unable to protect any citrate synthase activity from thermal inactivation.

Hsp27 protection of enzymatic activity

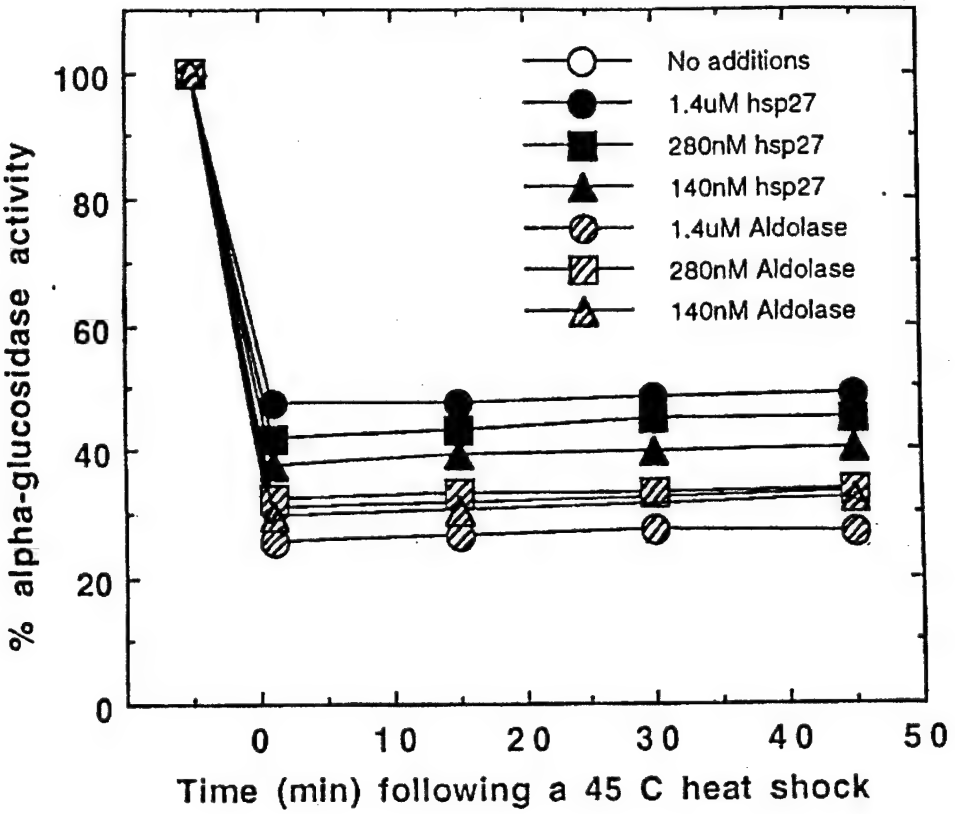

Fig 1

Hsp70 protection of enzymatic activity

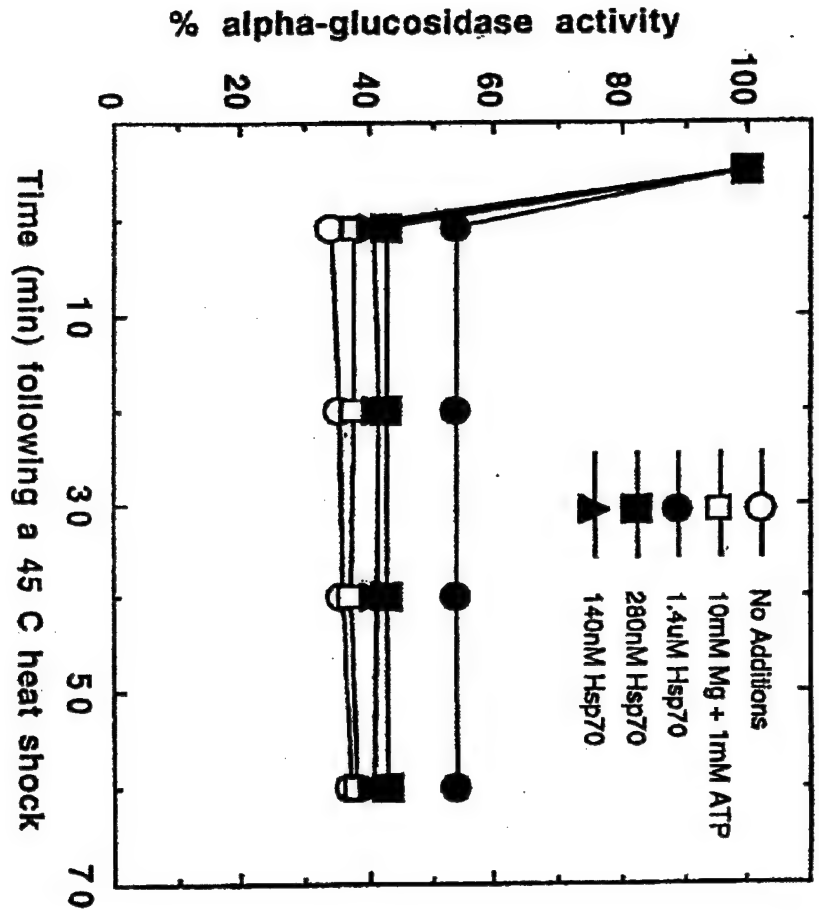

Fig 2

Hsp70 protection is ATP dependent

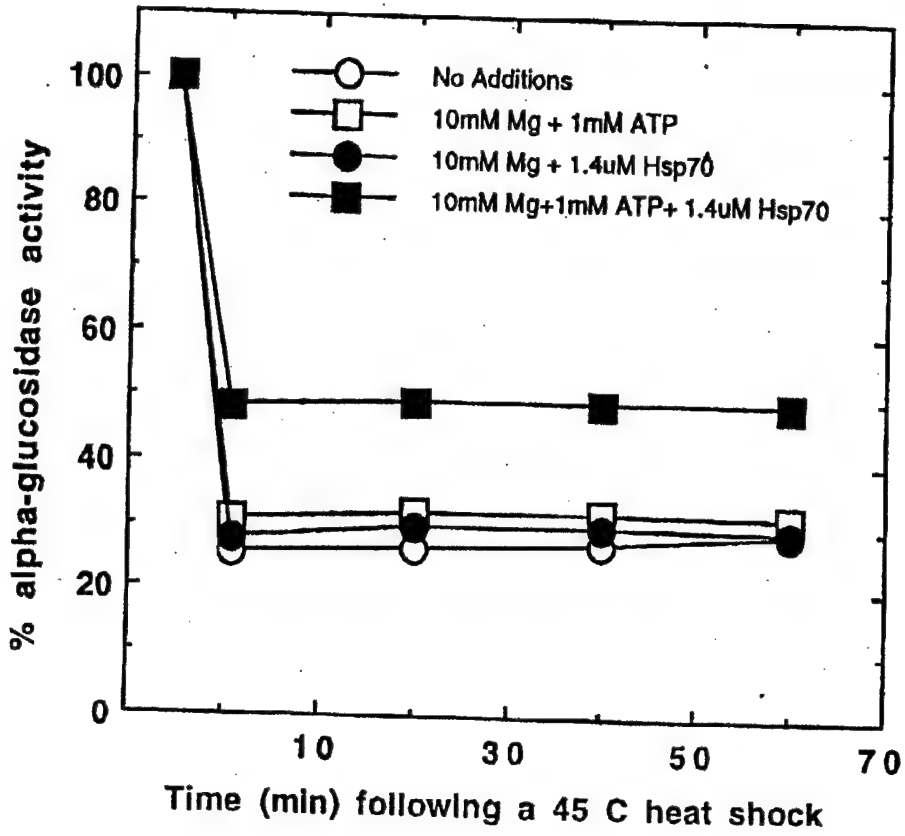

Fig 3

GroEL-GroES protection of enzymatic activity

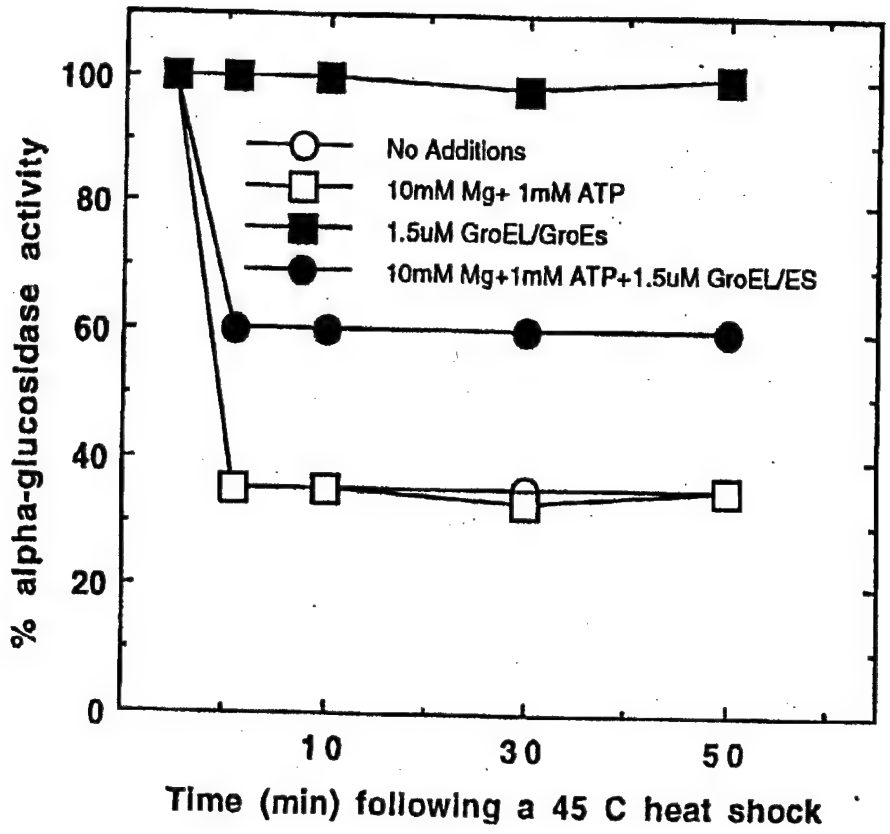

Fig 4

Citrate Synthase is protected by Hsp27

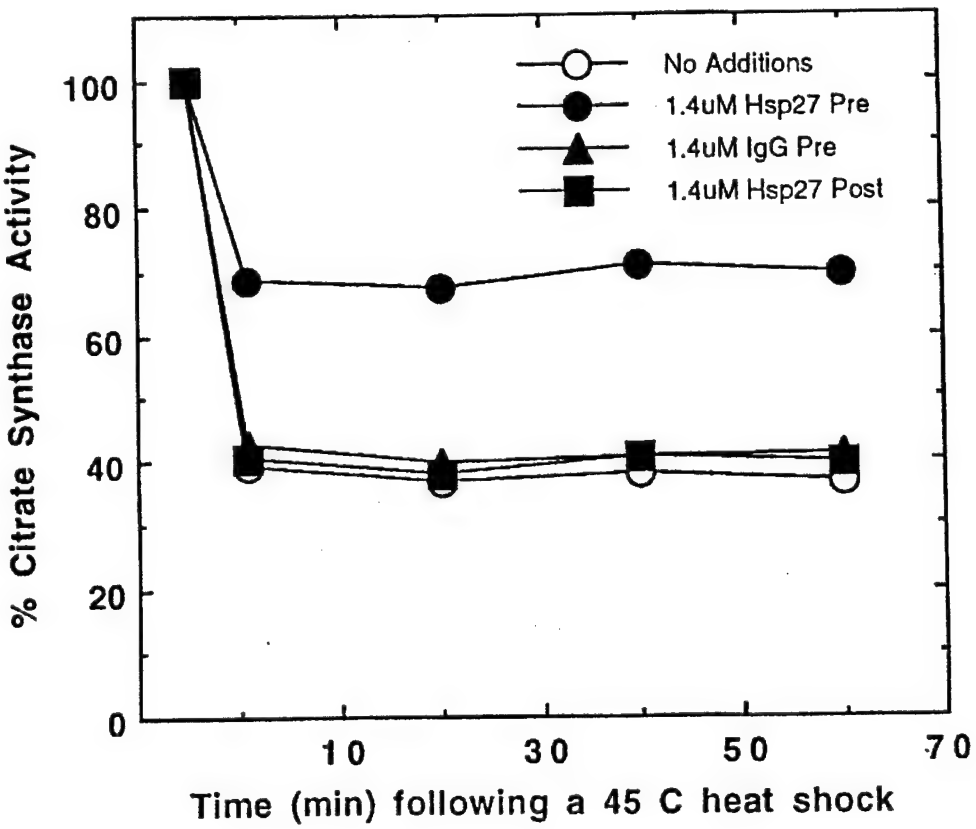

Fig 5

Citrate Synthase is protected by OAA

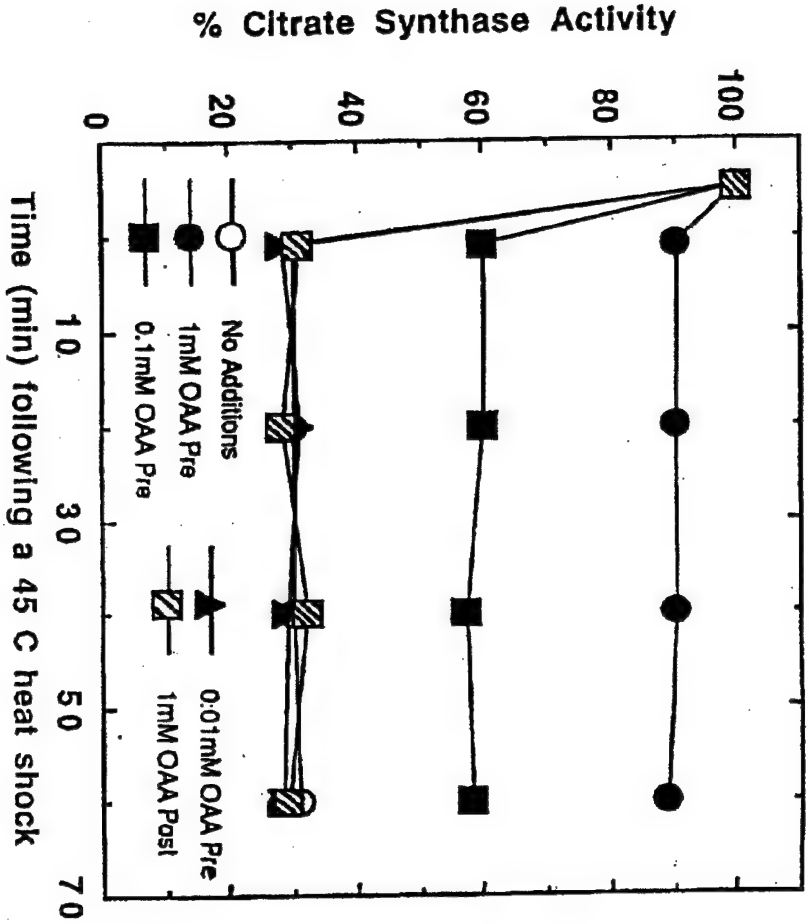

Fig C

Appendix E

Inhibition of Apoptosis by elevated levels of Heat Shock Protein 27 in Human Breast Cancer Cells

Inhibition of Apoptosis by elevated levels of Heat Shock Protein 27 in Human Breast Cancer Cells

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ABSTRACT

In Human breast cancer cells there can be elevated level of a stress response protein (Hsp27). Hsp27 has been shown to form an aggregate complex with cytochrome c. Caspase dependent apoptosis starts with the formation of the apoptosome and cytochrome c is one of the components of the complex. Because of the complex formed between Hsp27 and cytochrome c, the apoptosome may not form, therefore inhibiting the apoptotic cascade. In this experiment, it will be shown that the DB46 cell line which expresses elevated levels of Hsp27 has a reduced amount of apoptotic protease activity.

INTRODUCTION

Throughout a cell's life it receives many external signals that predicate its life expectancy or purpose. Certain signals will cause a cell to grow and divide, to differentiate into cells that make up a particular organ, and for apoptosis (programmed cell death).

Apoptosis is an important function of a normal cells life. There are several characteristics of apoptosis, which includes plasma membrane blebbing, nuclear condensation, loss of cell volume and DNA fragmentation (1). An extra cellular signal is given to a cell and it activates specific proteins that degrade genetic material, ultimately killing the cell. Apoptosis is commonly induced in embryonic development and tissue hemostats in eukaryotic cells (2).

Two major pathways are used for the induction of apoptosis. The receptor mediated apoptotic pathway (Figure 1, page 3) and caspase dependent apoptosis. There are two types of receptor mediated Apoptosis. The FAS receptor mediated apoptosis starts with the FAS ligand binding to the FAS receptor. The ligand/receptor complex will then activate FADD (FAS Associated Death Domain) which will cleave an inactive protease procaspase8, zymogen into its active form Caspase8. Caspase is a cysteine-requiring aspartate protease that is integral for the mediation of cell death (3). Caspase8 then activates Caspase3, an effector caspase which is responsible for the activation of other degradative proteins that destroy genetic material. A second receptor mediated pathway is the TNF-α (Tumor Necrosis Factor – Alpha). Again, the ligand binds to a receptor and this complex initiates the activation of FADD. Next, FADD activates Caspase8 from procaspase8 and finally Procaspase3 is activated and the cell death cycle has begun.

Caspase dependant apoptosis is the other pathway that plays an important role in understanding the experimentation in this paper (Figure 2, page4). An external stimulus is given to commit the cell to die and cytochrome c, (a mobile single electron carrier protein is used in the generation of cellular energy), is ejected from the mitochondria and released into the cytosol of the cell. Apaf-1 (Apoptotic Protease Activating Factor-1) then associates with cytochrome c in a dATP dependant complex called the apoptosome. The apoptosome then recruits procaspase9, because of its ability to recruit other caspases. When procaspase9 is bound to the apoptosome, it enhances its ability to cleave other caspases with a three fold increase in caspase3 cleaving catalytic activity (4).

Once the Apoptosome is formed a "cascade" effect activation of proteases is initiated. The caspase9 bound apoptosome then cleaves another zymogen, procaspase3 to caspase3. Caspase3 then degrades ICAD (Inhibitor of Caspase Deoxyribonuclease) that is a control point for the inactivation of CAD (Caspase Deoxyribonuclease). Once activated, CAD enters the cell's nucleus and destroys DNA, the genetic material necessary for replication.

A significant factor that differentiates normal cells from cancerous cells is the cells ability to evade apoptosis. Previous work in Dr. Carper's lab has shown various apoptotic inducing chemicals that were administered to DB46 (Human Breast Cancer cells with elevated levels of Hsp27) (Figure 3). The DB46 cells had a higher survival percentage when compared with DC4 cells (Normal cells with basal levels of Hsp27). This data suggests that a cells ability to escape

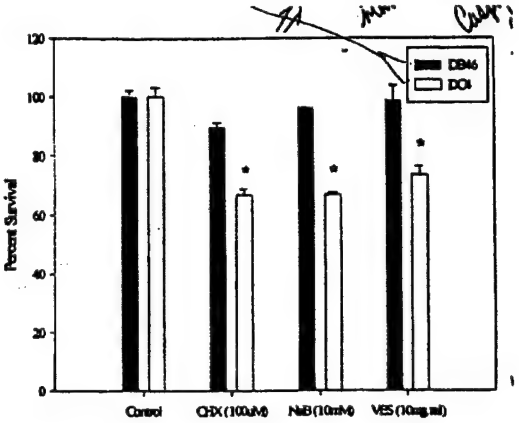


Figure 3. Apoptosis inducing drugs. 10mM Sodium Butyrate can induce apoptosis in DC4 cell lines.

apoptosis has to do with elevated levels of Hsp27 within the cell.

Heat Shock Protein 27 is a molecular chaperone protein responsible for helping other proteins obtain their three dimensional structure. It also is an estrogen responsive protein and at high levels of the hormone, Hsp27 will be expressed in elevated amounts. Heat Shock Proteins protect and repair damage that results from heat shock and oxidative stress (5, 6).

In cell free extracts (*in vtiro)* it has been shown that Hsp27 will form a complex with cytochrome c (7). This complex could be contributing to the inactivation of the apoptosome. This set of experiments will hypothesize that elevated levels of Hsp27 in human breast cancer cells does bind to cytochrome c and actually prevent the apoptosome formation, thus inactivating the caspase cascade in the actual cell lines DB46 and DC4 (*in vivo)*. Early studies have shown that cytochrome c does form a complex with Hsp27 (7). This conclusive data

could be the reasoning behind improper or inactive apoptosome formation, which allows the cell to evade apoptosis.

MATERIALS AND METHODS

Size Exclusion Chromatography – Size exclusion chromatography is also known as gel filtration chromatography or molecular sieve chromatography. The method is based on the ability of molecules to elute through a column. The bead material is made up of porous agarose beads that allow smaller molecular weight macromolecules to enter and larger ones to pass by. This separates the molecules on the basis of larger macromolecules elute first through the column because of little to no

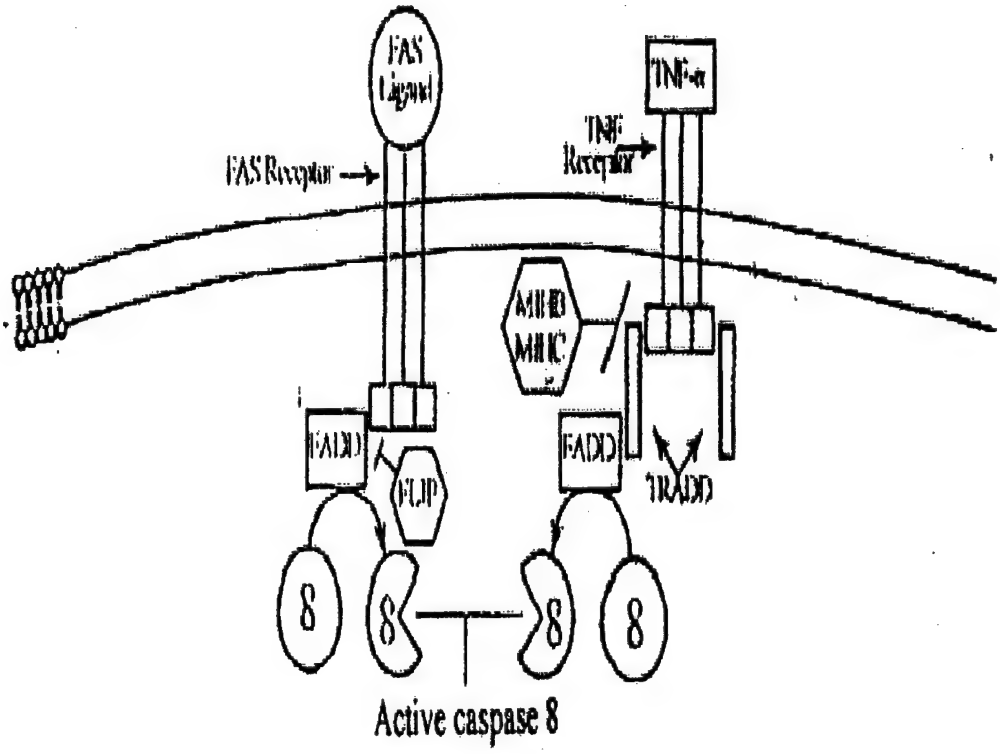


Figure 1. Receptor Mediated Apoptosis. On the left, FAS ligand binds to a FAS receptor causing a complex to form. This activated complex then associates and activates FADD (FAS Activating Death Domain), which in turn cleaves procaspase 8 to its active form caspase8. The right has a similar pathway but the difference is in the ligand and the receptor being from the TNF-α (Tumor Necrosis Factor Alpha) transmembrane protein family. Mammalian inhibitors of apoptosis homologs B & C, MIHB and MIHC will inhibit TRADD so that the cleavage of procaspase8 to caspase8 is not allowed. Adapted From: Joe Stafford's Thesis (1).

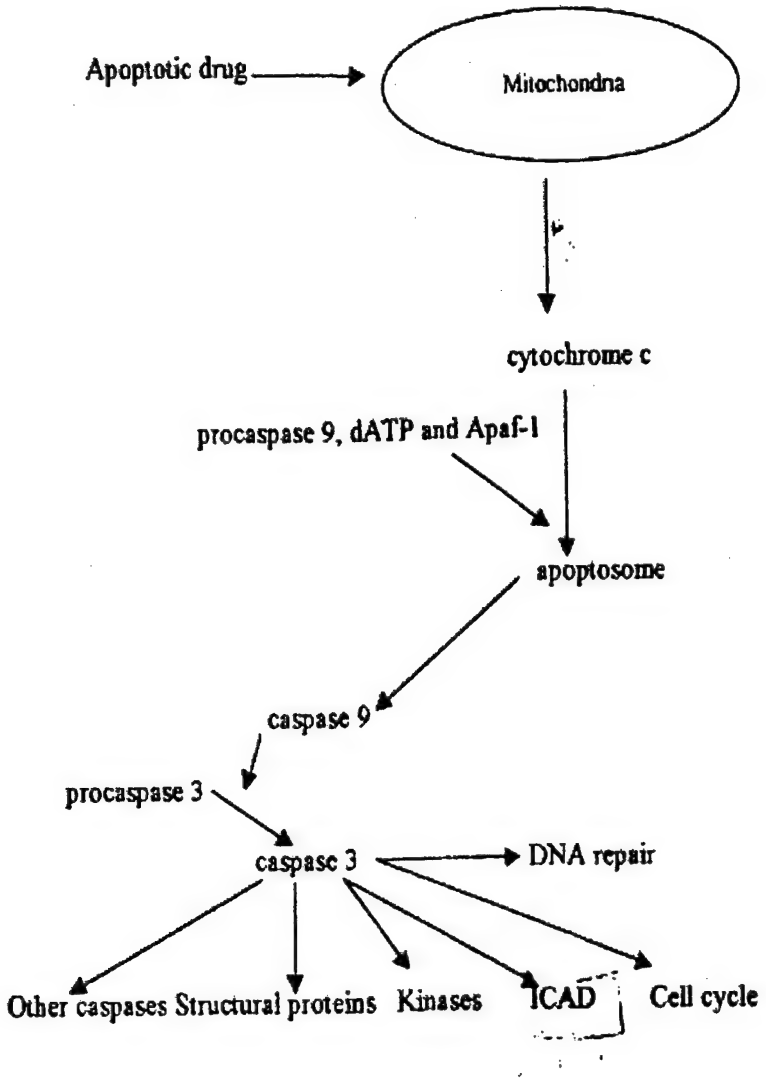


Figure 2. Caspase Dependent Apoptosis. An apoptotic drug is administered to the cell and the ejection of cytochrome c follows. This binds with procaspase9, dATP, and Apaf-1 to form the Apoptosome. Once the apoptosome is activated it can cleave proteases that ultimately lead to the destruction of the cell. Adapted from Joe Stafford's Thesis (1).

interaction with the porous beads and smaller macromolecules coming out last because of the proteins entering the beads thus slowing down overall migration through the column (Figure 4).

The column was prepared by using a plastic 10mL pipette and cutting off the top and bottom allowing the shell of the pipette to act as the empty column. Next, glass wool was inserted into the pipette to act as a barrier not allowing the porous beads to come out the bottom. Four centimeter rubber tubing was added to the bottom of the pipette so that a proper spout could be constructed from

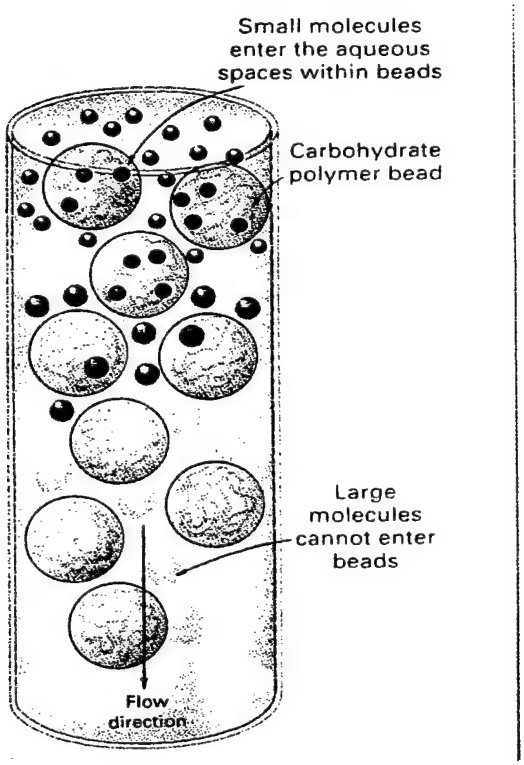


Figure 4. Gel Filtration Chromatography. The separation of three different proteins, the larger proteins emerge before the small proteins because of the interaction with column material.

a 200µl pipette tip. The column was then transferred into the cold room (4 C) that would ensure an optimal temperature for the proteins that were going to be administered into the column. The column was stabilized by a ring stand and clamps so that it would be completely vertical, thus allowing the proteins to consistently flow through the column. Initially, two columns made from SuperRose Grade 6 Prep (Sigma-Aldrich) were chosen because of their ability to differentiate between proteins of a wide variety of sizes. Fractionation range was between 5,000 Daltons (Da) and 5,000,000Da and the apoptosome were determined from previous literature at approximately 700,000Da (11), thus making the SuperRose material acceptable for this experiment. The beads are made from highly cross-linked agarose material and have a diameter of 20-40 µm. The beads were suspended in 20% ethanol. The eluent, or solvent used to allow proper migration of proteins through the column up was 40mM Hepes (N-2-hydroxyethyl piperazine-N'-2-ethane-sulfonic acid, ph 7.5) buffer. The final volume of material put into both the columns was 10mL of SuperRose Grade 6 Prep beads. Due to poor resolution of the SuperRose Grade 6 prep beads another column material was needed. SuperDex 200 Prep Grade beads (Sigma-Aldrich) were chosen due to good resolution from previous work done and availability of the beads on a short notice. The Superdex beads are different from the SuperRose beads because of the cross-linking of agarose and dextran. However, the fractionation range of the beads is much smaller at 10,000Da to 600,000Da and a bead diameter of 24-

44μm. So just from the approximation of the bead fractionation the apoptosome should elute out in the void volume, or the volume large proteins have no interaction with the porous beads. Two columns were constructed to save time and were prepared similar to one another. Approximately 11mL of bead material were pored into both columns. After 30mL of Hepes buffer was applied through the columns to remove all the ethanol, the color standards, protein standards and cell lysate was administered to the column.

Color standards are used to calibrate the column to determine visibly (due to the standards absorbing in the visible region, Blue Dextran is blue and DNP-glutamate is yellow) when the large macromolecule Blue Dextran (2,000,000Da) would elute out and when an extremely small molecule, DNP-Glutamate (300Da) would come out after interaction with the SuperDex beads. Five milligrams of Blue Dextran and one milligram of DNP-Glutamate were suspended in 1mL of Hepes buffer and 250μl were added to the column.

Protein Standards (Sigma-Aldrich) were made up of Thyroglobulin, Apoferritin, Bovine Serum Albumin, and Lyzozyme. All concentrations applied to the column were at 1 mg mixed with 1mL of Hepes Buffer. Then 250μl were administered to the columns and fractions consisting of 100μl, except for the void volume were taken.

After preparation of cell lysate 100μl of DB46 (induced into apoptosis using 10mM Sodium Butyrate), DC4 (uninduced-Control and induced using same procedure as the DB46) cell extract was administered to the column and 100μl fractions were obtained to find the fraction containing the apoptosome.

Protein Assays – Almost every protein method known exhibits a discrete sensitivity to the application of a specific biochemical reagent when reacting to the protein of interest (9). The BCA Protein Assay Reagent was chosen because of its ability to reduce Cu^{2+} using the protein of interest to Cu^{+} in medium that is slightly alkaline, (Pierce).

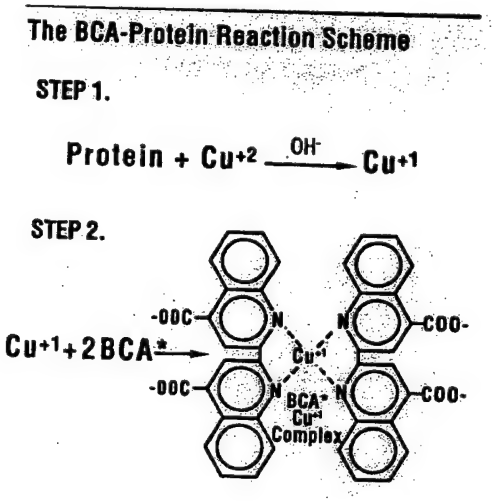


Figure 5. The involvement of Copper with Bicinchoninic acid when a protein interacts with the complex.

The reagent chosen is BCA, bicinchoninic acid, and this complexes with the proteins and allows the reduction of Copper, which causes an absorbance of approximately 562 nm in the visible region (purple).

Using a 96-well plate, each fraction off the column was put into a well at the volume of 50 μl. Hepes was used as the blank to zero the instrument

using the solvent which was ran through the column. A 50 parts to 1 part mixture of Reagent A to Reagent B, respectively was made and 200 µl of the mixture was added to each well. The plate was then mixed briefly and incubated for 30 minutes in a 37 C water bath.

After incubation the plate was read using the DYNATECH MR5000 plate reader. This passes light through the wells and based on the absorption in the visible region due to protein content will have various degrees of absorbance readings. Therefore the more protein in each well, the higher absorbance will be obtained. The samples were analyzed at 570nm, which is the closest to the maximum wavelength that the copper/BCA/protein complex absorbs.

For color standards, the standards were chosen because of their ability to absorb in the visible region. Using a 96-well plate, each well had 100 µl of color standard from the fractions collected off the column. The Dynatech MR5000 plate reader was used but the wavelengths were adjusted for maximum absorbance based on the Color Standard. Blue Dextran has a peak absorbance of 630nm and DNP-Glutamate has a maximum absorbance of 410nm.

Preparation of Cell Lysate – In order to get the proteins of interests into a form that would be appropriate to apply to the column for fractionation a multi-step process was formulated. First, the cells of interest DC4 and DB46 had to be grown specifically for this experiment. The growth phase had to be precise to allow the cells to be in a sub-confluent phase. This part of the growth phase is when proliferation of the cells is at its maximum and they aren't in a survival mode as they would be if they were confluent. Confluent cells have to deal with various external stimuli that induce them to grow in a "defensive" manner. Such stimuli include lack of growth space, deficiency in nutrients to grow and warding off toxic by-products.

Cells were allowed to grow 2-3 days after they were passed to new media and space in flasks. After the growth period, 1mL of trypsin was applied to the cells so that they are cleaved from their growth surface (The bottom of the flask). The trypsin was allowed to react for 3 minutes before 4mL of media was used to neutralize the trypsin. For cells that were induced to undergo apoptosis, a 1M solution of Sodium Butyrate (NaB) was made by mixing 1.1009g of NaB into 10mL of distilled water. Then 50µL was put into 5mL of cells to get a final concentration of 10mM NaB for optimal apoptotic induction (Figure 3, page 2). This was determined from previous studies done on apoptotic inducing drugs.

A period of 15 minutes was needed to have optimal Caspase9 activity, indirectly implying the activation of the apoptosome.

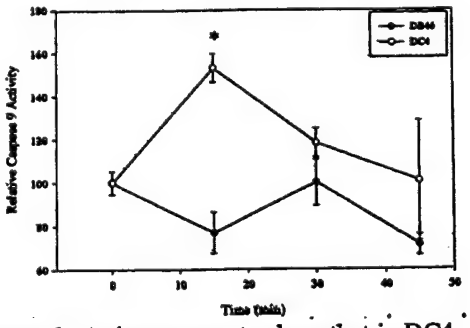


Figure 6. A time course to show that in DC4 cells the apoptosome will form because of the activation of caspase 9 at approximately 15 minutes.

Once induction of apoptosis has been done, the following procedure can be used for both induced and non-induced cells. Harvest all 5mL of cells after the media has been applied and count the cells using the Coulter cell counter to determine approximately how many cells are in 100μL of solution. Take cells and centrifuge them using the 4 C centrifuge.

Centrifuge instructions were as follows. Open the top of the centrifuge and put in samples making sure to counterbalance them with volume weight exactly the same amount as cell volume. Speed setting was anywhere between 4 and 5, which is 1000 to 2000 revolutions per minute. High brake allowing the quickest stop of the rotor once the material has been centrifuged. A time set of 15 minutes for appropriate sedimentation to occur.

The pellets were then taken and the solution was poured off, so that only cells were collected. The pellets were then resuspended using 5mL of Phosphate Buffered Saline (PBS). Lysis Buffer (1 times) was added at 25ul per million cells to ensure proper rupturing of cells in the sample. A second centrifugation was done using the exact procedure from above, but this time the supernatant or solution was collected because the protein of interest should be separated from the cell material and other large membrane organelles. All Cell extracts except for 5μL was added to the columns and fractions of 100μl were then collected.

Caspase3 Activity Assay – The theory behind the Caspase3 activity assay is the ability of the apoptosome to cleave Caspase3, by activation of Procaspase9 to Caspase9. Then Caspase9 can activate Procaspase3 to Caspase3 and the substrate of the Caspase3 activity assay should be cleaved into a detectable form.

The Caspase3 assay is based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp p-nitroaniline (Ac-DEVD-pNA) by the active form of Caspase3. This results in the release of p-nitroaniline (pNA) (3).

Ac-DEVD-pNA ~~~~> Ac-DEVD
+ pNA

The newly released p-nitroaniline has a maximum absorbance of 405nm and the DYNATECH MR5000 plate reader can be used to determine various concentrations of pNA in wells, if any, of a 96-well plate.

The reagents supplied were in excess so various dilutions and stock solutions were needed to be prepared. First an approximation for the amount of each substance was needed based on how many wells were going to be used.

1. Assay Buffer: Five mL was needed for all the appropriate dilutions and well volumes. Five hundred microliters of 10 times Assay buffer was added to 4.5mL of 17 megohm water (Distilled water).
2. Caspase Substrate: Dissolved 2 mg of substrate in 147μl DimethylSulfoxide (DMSO). Then add 1.323mL of 1 times Assay buffer for a 10:1 dilution of stock solution.

3. Caspase3 Positive Control: Reconstitute the vial of caspase3 enzyme (5µl) with 50µl of 17 megohm water. Store in 25 aliquots of 2µl of Stock at -70 C. Just before use, dilute an aliquot in 98µl of 1 times Assay buffer.
4. p-Nitroaniline standard for calibration curve: Dissolve the vial (1mg) in .72mL of DMSO. Store at -20 C. For working concentration take 2µl of Stock solution and add 98µl of 1 times assay buffer.
5. Lysis Buffer: Take 100µl of 5 times lysis buffer and add 400µl of 17 megohm water.

After stock and dilutions are made, a calibration curve needs to be made to measure the content of pNA that is cleaved when substrate is added to cell fractions that do have Caspase3 activity. Concentrations of 0, 10, 20, 50, 100 and 200 µM p-Nitroaniline were made by taking the working solution and adding 0, 1, 2, 5, 10 and 20µl to corresponding wells to make a final solution of 100µl per well using 1 times Assay buffer. Repeat in triplicate for more accurate statistics (10).

For all cell extracts of DC4 and DB46, 85µl of 1 times Assay buffer and 10µl of Caspase3 Ac-DEVD-pNA substrate for each fraction and mix into a 96-well plate.
The Caspase3 positive control is used just to make sure you have added in the appropriate amounts of reagents to ensure that the actual experiment, under perfect conditions will actually work. Take 5µl of the Caspase3 positive control, 85µl of 1 times Assay buffer and 10µl of Caspase3 substrate into 3 wells of the 96-well plate for triplicate results.

Once all the wells have been appropriated with the corresponding mixtures read the plate using the DYNATECH MR5000 plate reader at 410nm.

RESULTS AND DISCUSSION

Choosing the best material column for the experiment – When the experiment began, the proper column material was needed to best resolve other proteins from the protein complex of interest, the apoptosome. From earlier journal articles the functionally active apoptosome has a molecular weight of approximately 700,000Da or 700kDa (11). With this initial starting point, the column material selected would be something that could resolve a protein complex of this size from other proteins. SuperRose Grade 6 beads (Sigma-Aldrich) was chosen due to its rather broad fractionation range of 5kDa to 5000kDa. Two columns were constructed and multiple runs of protein standards were conducted to determine the degree to which the columns could separate known molecular weight standards. Blue Dextran (2,000kDa), Thyroglobulin (443kDa) and Bovine Serum Albumin (66kDa) were chosen because of their various molecular sizes. Due to the poor resolution of the columns (figure 7) on numerous trials the decision was made to try for a better resolving column material.

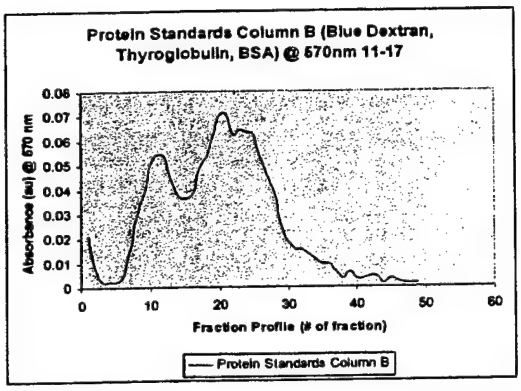
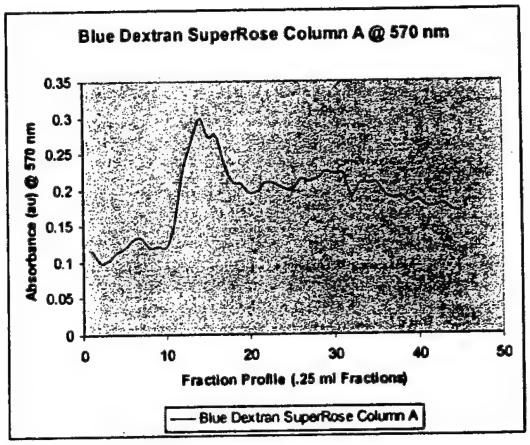


Figure 7. An example of the poor resolution of the SuperRose Grade 6 prep material using Blue Dextran (peak at fraction 12, Thyroglobulin (fraction #21), and BSA (fraction #26).

From past experiments and actually hands on experience, SuperDex200 Grade beads had a very applicable resolution, but due to the fractionation range of 10kDa to 600kDa is wasn't chosen because it could not properly resolve proteins of approximately 700kDa. Because of its ability to separate smaller proteins than the apoptosome, the SuperDex200 grade was chosen because it could separate the apoptosome from other caspases, so that any activity coming from the fractions of interest must be the apoptosome activated complex. Procaspase9 forms an active dimer at approximately 90kDa and Procaspase3 forms an active heterotetramer composed of two large subunits and two small subunits at about 60kDa (11). Two columns with the SuperDex200 Grade prep were constructed. This allows us to differentiate between caspases and the apoptosome. To compare the type of resolution that each column had, 5mg of Blue Dextran was dissolved into 1mL of Hepes and then 500µl were added to each column (Figure 8).



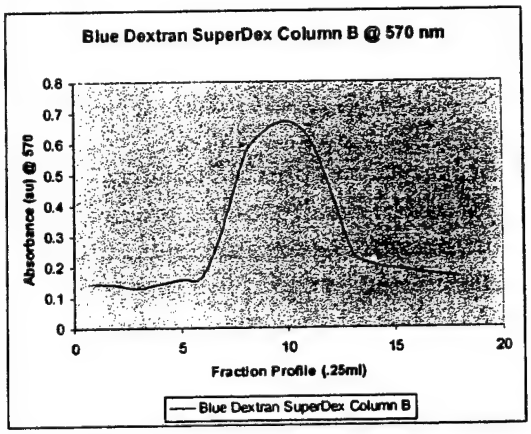


Figure 8. Comparison between Column Resolution using the same concentration of Blue Dextran. SuperRose (top) and SuperDex (bottom) column materials.

Void Volume and Total Column Volume – Two colored molecules were chosen for the protein standards to visibly detect when the color standard is going to migrate out of the column and to determine the void volume, or total space surrounding the beads in a packed column (12). Blue Dextran (2,000kDa) and DNP-Glutamate (0.3kDa) were chosen due to the large difference in molecular weight.

After the fractions were collected and analyzed a graph was constructed to

show the relationship between absorbance and fraction number (Figure 9, 10). Blue Dextran eluted out at fraction number 11 and DNP-Glutamate eluted out at 51 using 630nm and 410nm respectively to obtain peak absorbance.

The total volume was added up due to the first couple of fractions were taken at 1mL instead of 100µl. The void volume was determined to be 5.1mL. The end volume or volume when DNP-Glutamate migrates out at was experimentally found to be 9.1mL. Therefore subtracting the end volume from the void volume, the total column volume can be determined for both columns due to similar column material (11.0mL). Total column volume for both columns was found to be 4.0mL.

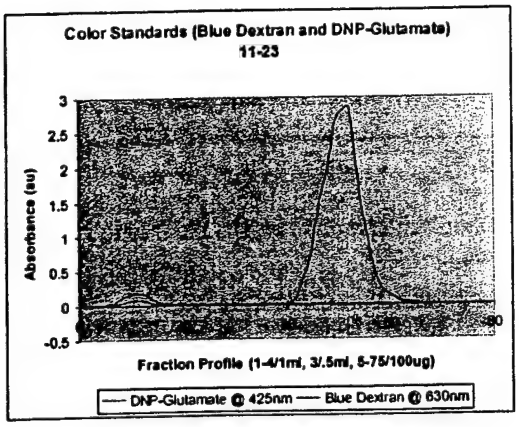


Figure 9. Color Standards (Blue Dextran and DNP-Glutamate) ran at 630 and 410nm respectively for peak absorbance.

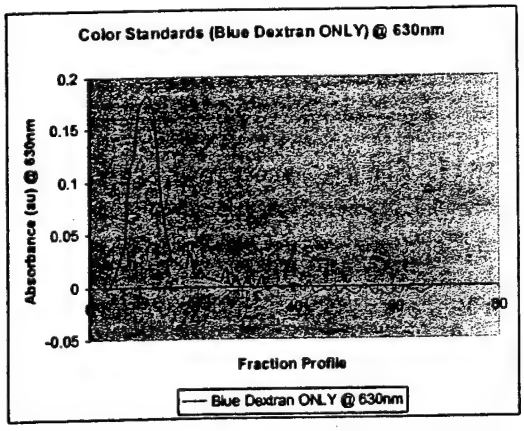


Figure 10. The single peak of Blue Dextran so it can be actually seen as a peak and not being compared to the larger DNP-Glutamate peak.

Protein Standards – Thyroglobulin, Apoferritin, Bovine Serum Albumin (BSA) and lyzozyme were used as the Protein standards in both columns (B, C). Consistent with gel filtration chromatography the largest protein, Thyroglobulin (669kDa) came out first; followed by the next largest, Apoferritin (443kDa); next, BSA (67kDa); and finally lyzozyme (27kDa) Figure 11, 12.

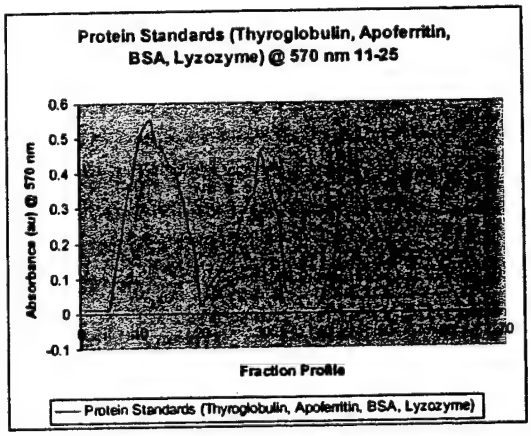


Figure 11. Column B Protein Standards (Thyroglobulin, Apoferritin, Bovine Serum Albumin (BSA), and Lyzozyme.

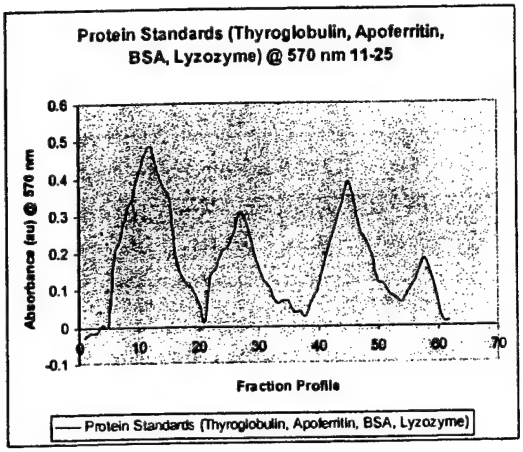


Figure 12. Columns C Protein Standards (Thyroglobulin, Apoferritin, BSA, Lyzozyme) read at 570nm using BCA protein Assay.

The peaks did not come out perfectly symmetrical, but the similarities in peaks shows some consistency with the proteins that are migrating through the column. A superimposition of the peaks has been done to show that the fraction numbers at which the proteins elute out are consistent as well as the peak asymmetry (Figure 13).

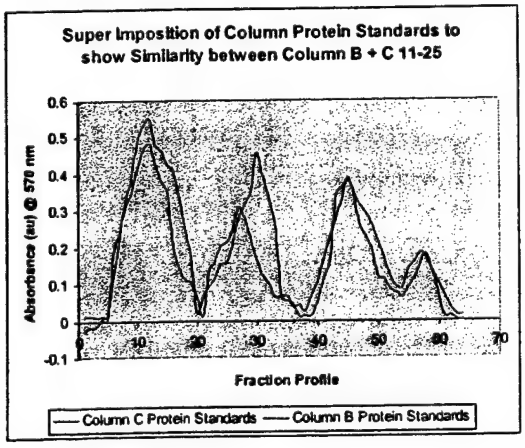


Figure 13. Superimposition of Columns B and C to show similarities between columns and the asymmetry of the isolated peaks.

Next a graph of the log of the molecular weight versus the fraction number was obtained to plot the known weight of the apoptsome, Caspase9 dimer and Caspase3 heterotetramer.

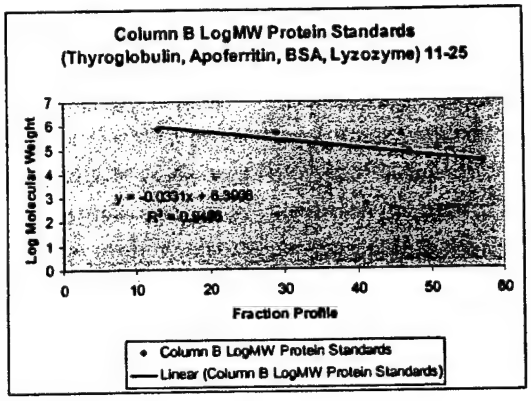


Figure 14. . Column B linear graph of the log of the molecular weight versus the fraction number.

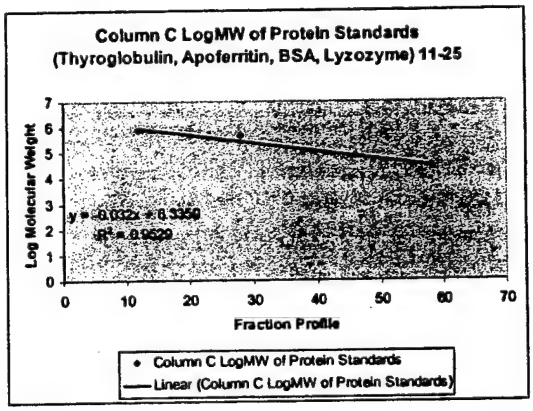


Figure 15. Column C linear graph of the log of the molecular weight versus the fraction number.

The protein standards peak absorbance was determined by finding the maximum absorbance and correlating that to a fraction number. For column B, thyroglobulin (fraction number 13), apoferritin (fraction number 29), BSA (fraction number 47) and lyzozyme (fraction number 57) were

determined as the peak absorabances. With a linear equation of y = -.0331x + 6.3908 and an R^2 value of .9468, which corresponds to approximately 94% of the sample variation can be explained using the straight line model (10). For column C, thyroglobulin (fraction number 12), apoferritin (fraction number 28), BSA (fraction number 46) and lyzozyme (fraction number 58) were determined. The linear equation of y = -.032x + 6.3359 and a R^2 of .9529 were obtained, stating this graph has a better chance of finding the actual fraction of the Apoptosome when compared to column B. Also, a superimposition of both linear graphs was done to show the similarities in the Columns (Figure 16).

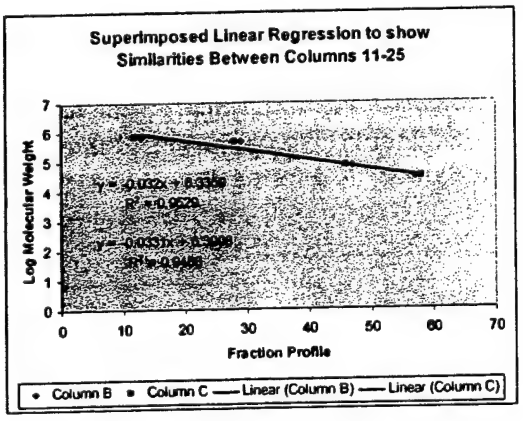


Figure 16. The superimposition of the log of the molecular weight versus the fraction number for columns B and C.

Determination of the Apoptosome, Caspase9 and Caspase3 Fractions – Using the linear equation graphs from figure 16 and the linear equations derived from those graphs, the fraction number of the apoptosome, the active Caspase9 dimer and the active Caspase3 heterotetramer can be determined.

The log of molecular weight for the apoptosome (5.8451) was determined by taking the log of 700,000Da. For Caspase9 the log of the molecular weight is (4.95424) for a 90,000Da dimer and Caspase3 is (4.7782) for the 60,000 Heterotetramer.

Using these values and substituting them for y in the linear equation the x, or fraction number can be obtained. For Column B, the apoptosome elutes out at fraction 16, Caspase9 at fraction 43 and Caspase3 at fraction 49. For Column C, the apoptosome comes out at fraction 15, Caspase9 at fraction 43 and Caspase3 at fraction 49.

Cell Lysate Analysis – All procedures were followed in the Material and Methods section, but a couple of unforeseen errors occurred. One, the pellet from spinning down the 5mL of cell solution was almost non-existent, so that when the solution or supernatant was discarded hardly anything remained for resuspension using Phosphate Buffered Saline. This lead to the next problem, which was the addition of 1mL of PBS for every 1 million cells, because of the cell count being low (DC4 uninduced at 322,000 per flask and DC4 induced at 360,000 per flask) so the addition of 400µl of PBS was added. Then 25µl of lysis buffer was added to complete the homogenization of the cells.

Caspase3 Activity Assay – Once the cell fractions were collected from the gel filtration chromatography, the Caspase3 activity assay could be introduced.

Taking 50μL of each fraction and putting them into a 96 well-plate leaving the top row open to allow for a blank, which consisted of 50μL of Hepes, all the following reagents were added to complete the assay requirements: 50μL of assay buffer plus 10μL of caspase3 substrate.

A calibration curve for p-nitroaniline was constructed using various increasing concentrations. 0, 10, 20, 50, 100, 200μM were used to find the range of pNA cleaved in each well. They were diluted using Hepes buffer up to a final volume of 100μL. This was done to determine the amount of actual caspase3 activity in any given sample (Figure 17). This was done in triplicate to reduce the amount of error introduced due to poor technique and micromolar concentrations. Also a caspase3 positive control was done in triplicate and consisted of 15μL of caspase3, 85μL of Hepes buffer, and 10μL of caspase3 substrate to ensure that the assay was actually working.

The calibration curve had a linear equation that was $y = .0009x + .0025$ with a R^2 value of .9939, which suggests that proper dilution factors and technique lead to a graph that will be correct when finding the unknown concentration 99% with each trial. The calibration curve for the uninduced was done also, but because of no activity it wasn't used and thus not presented.

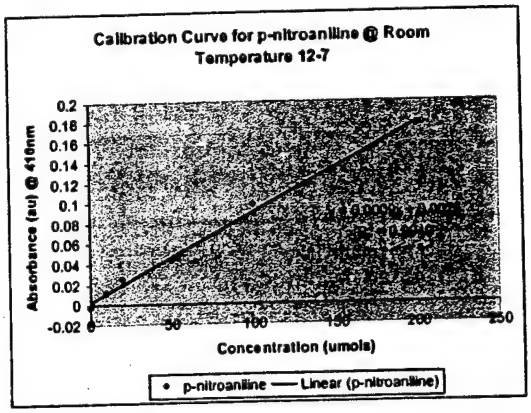


Figure 17. Calibration Curve of p-nitroaniline for the DC4 induced cell extracts at 0, 10, 20, 50, 100, and 200μL.

Once the 96-well plate was read using the MR5000 DYNATECH plate reader a graph of the DEVD-pNA or caspase3 activity versus the fraction number could be constructed for both the DC4 uninduced and induced cell extracts (Figure 18, 19).

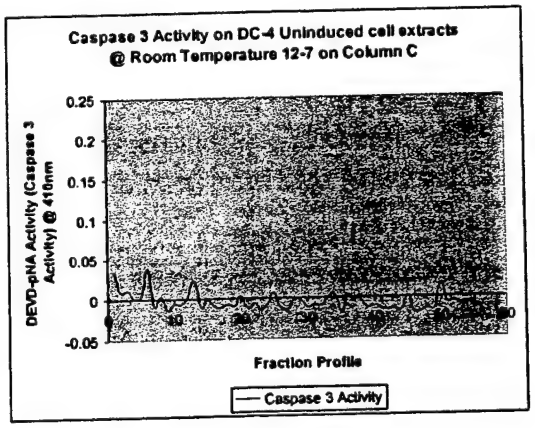


Figure 18. Caspase3 activity assay for DC4 uninduced cell extracts. No relative activity.

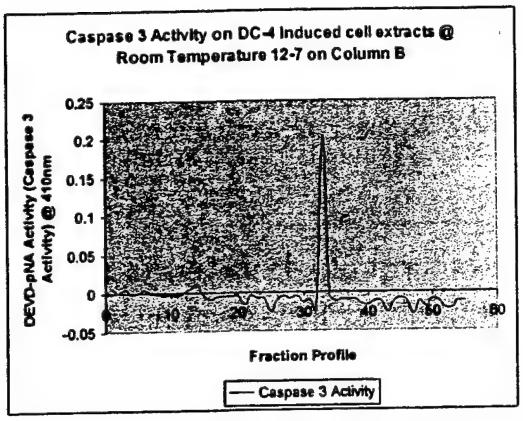


Figure 19. Caspase3 activity assay for DC4 induced cell extracts. One sharp peak around fraction 32.

As stated earlier in the introduction, the DC4 uninduced cell extracts should have no caspase3 activity due to the fact the apoptosome has not formed because there was no commitment to cell death, but as expect in the DC4 induced cell extracts there was a sharp peak around fraction 32, corresponding to a molecular weight of approximately 118kDa. The peak had an absorbance of .202au and using the calibration curve of pNA for the DC4 induced 96 well-plate it was determined to have a concentration of 222μM of cleaved substrate p-nitroaniline.

The same procedure was used for the DB46 cell extracts except for the fact that the cell concentration that was applied was different from the concentration used in the DC4 experiments.

A calibration curve and a caspase3 positive control were also done in triplicate to keep technique consistent. The calibration curve (Figure 20, 21) for DB46 uninduced had a linear equation of $y = .0007x + .0059$ and a R^2 value of .9885. For the DB46 the calibration curve had a little more error with a linear equation of $y = .0004x - .0121$ and a R^2 of .9432.

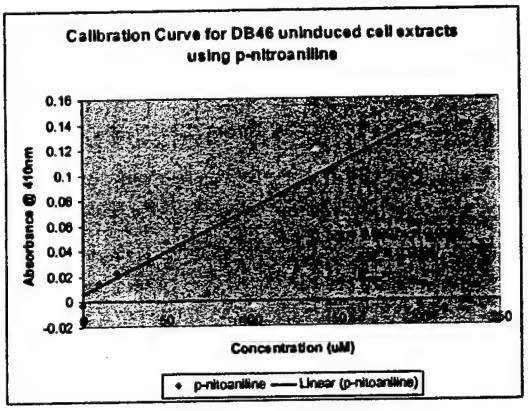


Figure 20. Calibration curve of p-nitroaniline at 0, 10, 20, 50, 100, and 200μM for the DB46 uninduced cell extracts.

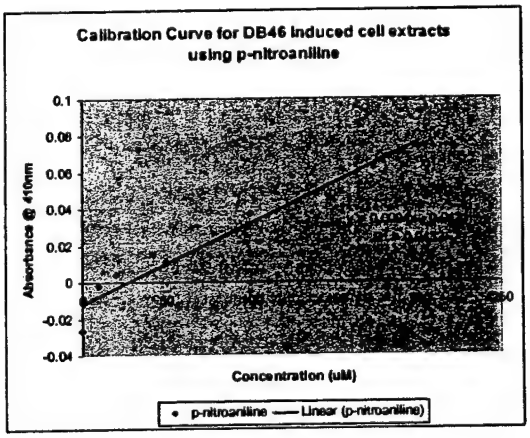


Figure 21. Calibration curve of p-nitroaniline at 0, 10, 20, 50, 100 and 200μM for the DB46 induced cell extracts.

When the graphs of the DB46 uninduced and induced were constructed they also had results consistent with the evidence that hsp27 does inhibit apoptosis (Figure 22, 23). The elevated levels of Hsp27 should bind cytochrome c when ejected out of the mitochondria. This prevents apoptotic activation, thus

little to no caspase3 activity should be present in either of the DB46 graphs.

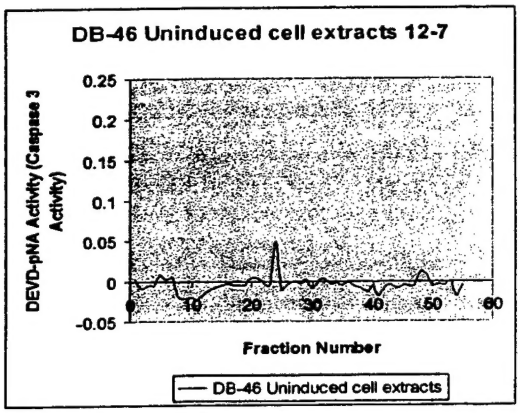


Figure 22. Caspase3 activity assay for DB46 uninduced cell extracts. One sharp peak approximately at fraction 24.

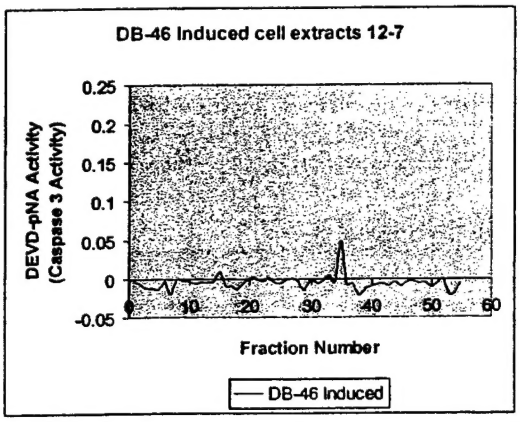


Figure 23. Caspase3 activity assay for DB46 induced cell extracts. One sharp peak approximately at fraction 34.

The DB46 uninduced had some unpredicted results. There was a small peak at fraction 24, which correlates to a molecular sized protein of approximately 190kDa; however with an absorbance of .048 that corresponds to a concentration of 60μM, which is consistent that there should be reduced activity, even when there was twice the concentration of cell extracts added to the columns when compared to the DC4.

The DB46 induced had a small, sharp peak at fraction 34, which has a molecular weight of 111kDa according to the log of the molecular weight vs. the fraction number linear equation. It had an absorbance of .049 that relates to a 152μM concentration of the product. As stated earlier the DB46 cell extracts should have reduced activity signifying the binding of cytochrome c with hsp27 causing reduced apoptosome formation and caspase3 activity.

CONCLUSIONS

When looking at all the data superimposed on one graph to compare peaks there can be assumptions made on the reasons for peak formation in the DB46 cell extracts (Figure 24).

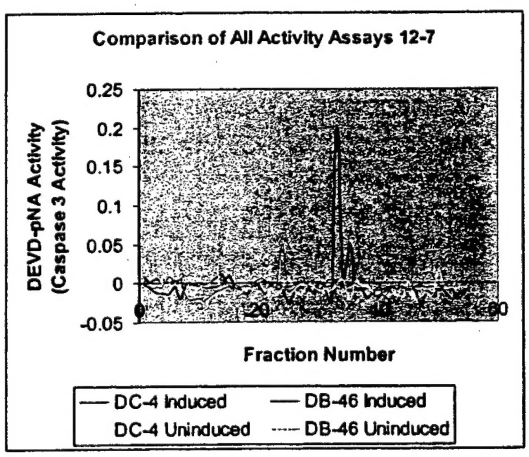


Figure 24. Superimposition of all Caspase3 activity assays. DB46 (uninduced/induced) and DC4 (uninduced/induced).

Once molecular weights of proteins with caspase 3 activity were determined, previous literature needed to be referenced to see if there was any

correlation to the peaks in the experiment when compared to similar experiments done in journal articles.

In *The Journal of Biological Chemistry*, 2000 a journal article by Benedict *et al.* was extremely similar to this experiment. Using SuperDex200 column material followed by a caspase3 assay they determined enzymatic activity in 2R3T human kidney cells.

The peak observed in the DC4 induced cells at fraction 32 corresponded to a molecular weight protein of 118kDa. This is consistent with the model that an apoptosome is formed with administration of a cytotoxic drug (10mM NaB) and there should have been caspase3 activity because of activation of procaspase3 to active caspase3 by the apoptosome containing active caspase9. Referencing the journal, they observed a peak at about 95kDa that could be the active dimer form of caspase9. The discrepancy in molecular weight could be due to the fact that the two cell lines are different, human breast cancer cells compared to human kidney cells and the enzymes could be isoforms that differ only in molecular weight but reserve the same function. A more conclusive reason would be that it could be a complex of two heterotetrameric caspase3 molecules that would have a molecular weight of 120kDa. Because of the specificity of caspase3 to bind to a caspase3 substrate it could lead to a much larger absorbance when compared to the specificity of caspase9 binding to a caspase3 substrate. This is evident when comparing this peak, which is believed to be caspase3 to the other peaks that are most likely caspase9 activity.

For the DC4 uninduced cell extracts the observations were consistent with the model of apoptosis. When no cytotoxic drug is administered to the cell extracts there should not be any reason for the cell to induce apoptosis, thus killing itself. As the data showed there was no significant caspase3 activity, which confirms no apoptosome formation.

The DB46 uninduced cell extracts had one peak of interest at 24, which was an unknown protein or protein complex with a molecular weight of 190kDa. Following the model of apoptosis for cells with elevated levels of hsp27 this was an unpredicted result. It could be due to two factors, one that the peak is an artifact, an error due to only one trial of the experimentation and no other trial to verify that it wasn't from improper technique. Two, that it was an actual peak due to a protein with the ability to cleave the caspase3 substrate, thus releasing pNA and an absorbance could be read. Because the experiment was done at room temperature this could mean it was some other protease with aspartic acid cleaving abilities, because of the fraction number the protein was too small to be considered the apoptosome and too big to be a caspase 3 or 9 active molecule. When compared to Benedict's article they had a similar peak at 180kDa and stated that this could be a complexed form of two active caspase9 molecules giving it an overall weight of 190kDa, which is consistent with the data in this experiment. Also, because of the smaller peak when compared to the DC4 cell lines, this suggests it was caspase9 because it has less substrate specificity than caspase3. This would give the overall pNA cleaved

a reduced absorbance rate due to less molecules being altered.

Lastly the DB46 induced cells had data somewhat consistent with the model of caspase dependent apoptosis. We can speculate the peak is around the same fraction number as seen in the DC4 induced cell extracts, but it is reduced due to the fact that cytochrome c has bound to hsp27, not allowing the formation of the apoptosome. Although, not all cytochrome c were bound to hsp27 and a few actually made the apoptosome giving a relatively small caspase3 activity absorbance. We could also say that since the molecular weight is close to 95kDa, it is the active dimer form of caspase9 and is a different peak than the one observed in the DC4 induced cell extracts.

In any case, this data gives us information on what to do next to be more conclusive in the studies of hsp27 and its ability to inhibit apoptosis in human breast cancer cells. Possibly a Western Blott technique along with a caspase9 Activity assay would help getting more verifiable results in upcoming experiments. Also, the experiment will be moved back into the cold room to reduce other protease activity that could actually degrade the apoptosome and make all future room temperature experiments futile.

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